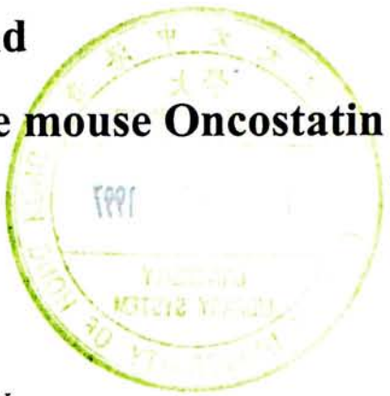
Cytokines and cytokine receptors expression profile during mouse embryogenesis and the molecular analysis of the mouse Oncostatin M gene



by

Miss Pui-kuen LEE

Thesis submitted as partial fulfillment

for

the degree of Master of Philosophy

August, 1996

Division of Biology

Graduate School

THE CHINESE UNIVERSITY OF HONG KONG

Statement

All the experimental work reported in this thesis was performed by the author, unless specially stated otherwise in the text.

Pui-kuen LEE

Acknowledgment

First of all, I would like to express my heartfelt thanks to my supervisor, Dr M. C. Fung. His patient guidance and invaluable advice were indispensible in the completion of this thesis project. I'm also indebted to his encouragement, inspiration and assistance in many aspects. My gratitude extends to the members of my thesis committee, Dr Vincent E. C. Ooi and Dr H. S. Kwan, for their patience in reviewing my thesis and their constructive comments. I am also grateful to Dr K. K. Mark for being my former marker. His enthusiasm and kindness are memorable.

Mr Nicholas S. H. Lai, Mr Arthur K. K. Ching, Mr K. S. Cheung, Mr Y. H. Yam were acknowledged for their valuable discussion on both philosophical and scientific issues. I also appreciate Miss F. Liu, Mr J. Wang, Miss Denise F. Y. Ho and Mr Stanton H. L. Kok for their excellent technical assistance. The colleagues in my laboratory, Miss Irene Y. Y. Szeto, Miss Pandora M. C. Yip, Miss S. J. Chapman, Mr Jack C. S. Lo and Mr W. L. Ng, should be mentioned, especially the harmony and humor they created. I would also like to thank many other colleagues in the department, they are Miss Polly P. Y. Cheung, Miss Renee Wu, Miss Debera K. Y. Fung, Miss Peggy B. H. Fung, Miss Iris S.W. Tong, Miss B. S. Ko, Miss W. J. Xie, Miss Annie S. C. Law, Mr Clive S. C. Lo, Mr Steven H. K. Chung, Mr Lawrence C. M. Chiu, Mr Clinton Y. H. Wong and Dr P. C. Leung,....etc., impossible to name all. Their precious friendships brought me encouragement and good memories.

I want to express special thanks to my parents for their love, care and forgiveness of my absences during numerous weekends of intensive work. Finally, my sincere gratitude goes to Mr Stephen H. W. Mok whose continuous, committed backup and spiritual support have meant so much to me in the course of my work.

Abstract

Cytokines are a heterogeneous group of polypeptide mediators that have been associated with cell-to-cell communication. During development, cytokines may act as direct intercellular signals by regulating proliferation and/or differentiation of competent target cells or as indirect signals by inducing the expression of a cascade of other genes which in their turn affect specific developmental events. However, the function of cytokines in embryogenesis still remains largely circumstantial and unexplored. In order to determine the *in vivo* embryonic role of cytokines, the first step is to determine when they are expressed during the life cycle of the mouse.

In the first part of the thesis, the expression patterns of 30 cytokines and cytokine receptors during mid- to late-gestation (7.5 to 17.5 d.p.c.) of ICR mouse were investigated using Reverse Transcription Polymerase Chain Reaction (RT-PCR). This method allows the detection and semi-quantitative assessment of changes in the mRNA level of various genes at different stages of development. The results revealed that more than half of the investigated cytokines and cytokine receptors had regulatory patterns among the 5 developmental stages, underlying certain biological functions in regulating proper embryonic development. However, most cytokines which were well documented to be important in adult hematopoiesis, like GM-CSF, G-CSF, IL-3 and IL-6, were absent from those embryonic tissues studied in this project. This observation indicates that the subset of cytokines controlling hematopoiesis in adult is different from those functioning in embryo. The absence of a number of seemingly essential cytokines in developing embryos proposed a hypothesis that there might be other not yet discovered cytokines specially functioning in embryos. Nevertheless, further investigation is required to support this hypothesis.

In the second part of the study, our focus turned to a novel cytokine, Oncostatin M (OSM), which belongs to the growing neuropoietic family. OSM, like

LIF and CNTF, was demonstrated to inhibit differentiation of pluripotential embryonic stem cells. This property implicates its role in development. Human OSM gene has already been cloned and sequenced for a long time but, interestingly, the mouse counterpart was not studied at the commencement of the present project. Therefore, a preliminary molecular analysis on mouse OSM gene was carried out to check the feasibility of using the coding region of human OSM gene as probe to isolate OSM gene from the mouse genome.

An OSM homologue was detected in mouse genome using hOSM exon 2 fragment as probe, however, the signal was very weak. Using hOSM exon 3 fragment as probe, on the other hand, resulted into smearing signals. The high G-C content in exon 3 coding region probably caused the non-specific binding. These findings concluded that isolation of murine OSM by cross-species hybridization was proven to be technically difficult.

Table of contents

Abbreviations

bFGF	basic Fibroblast growth factor
cDNA	complementary DNA
CNTF	Ciliary neurotrophic factor
CNTFR	Ciliary neurotrophic factor receptor
d.p.c.	Day postcoitum
DEPC	Diethylpyrocarbonate
DNA	Deoxyribonucleic acid
dNTPs	Deoxyribonucleotide triphosphates (dATP, dTTP, dGTP, dCTP)
DTT	Dithiothreitol
EDTA	Ethylenediamine tetraacetic acid
EGF	Epidermal growth factor
EtBr	Ethidium bromide
EtOH	Ethanol
G-CSF	Granulocyte-colony stimulating factor
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GM-CSF	Granulocyte-Macrophage-colony stimulating factor
hOSM	Human oncostatin M
IFN	Interferon
IGF	Insulin-like growth factor
IL-	Interleukin- (eg: IL-1 stands for interleukin-1 and so on)
IL-1R tI	Interleukin-1 receptor type I
IL-1R tII	Interleukin-1 receptor type II
IL-2Rα	Interleukin-2 receptor alpha
IL-3R(AIC2A)	Interleukin-3 receptor subunit (AIC2A)
IL-3R(AIC2B)	Interleukin-3 receptor subunit (AIC2B)
kb	Kilo-base pairs
LIF	Leukemia inhibitory factor
LIFR-l	Leukemia inhibitory factor receptor-long form
LIFR-s	Leukemia inhibitory factor receptor-short form
M-CSF	Macrophage-colony stimulating factor
MHC	Major histocompatibility complex
mOSM	Mouse oncostatin M
mRNA	Messenger RNA
NGF	Nerve growth factor
OSM	Oncostatin M
OSMR	Oncostatin M receptor
PBS	Phosphate buffered saline, pH7.4
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
RNA	Ribonucleic acid
RT	Room temperature
RT-PCR	Reverse transcription polymerase chain reaction
SCF	Stem cell factor

SCF-l	Stem cell factor-long form
SCF-s	Stem cell factor-short form
SDS	Sodium lauryl sulfate (Sodium dodecyl sulfate)
TE	Tris-EDTA
TGF	Transforming growth factor
TNF	Tumor necrosis factor
Tris	Tris (hydroxymethyl) aminomethane
UV	Ultraviolet
v/v	volume by volume
w/v	Weight by volume

List of Figures

List of Tables

Chapter 1

Introduction and Background

1.1 Role of cytokines in mouse embryonic development

1.1.1 Why mouse model

Genetic analysis of mammalian development has long been a challenge to scientists. However, the progress in mammalian study can never be as rapid as with *Drosophila* and *Caenorhabditis* (Hogan *et al.*, 1994), not only because of the large mammalian genome size and their long generation time but also due to the slow development of their embryos.

Of the diversity of mammals, the mouse has the advantages of small size, resistance to infection, large litter size and a relatively rapid generation time. The use of mice is also favored by the ease of availability and the simple breeding procedures. Therefore, the mouse has become firmly established for long time as the primary experimental mammal. It is also the organism on which most molecular biology and genetic experiments have been based.

1.1.2 Embryonic development of mouse

Embryonic development of the mouse begins with fertilization of the egg by the sperm. The method for timing pregnancy and the age of embryos used in this thesis followed Hogan *et al.* (1994). It assumes that fertilization takes place around midnight

on a 7 pm - 5 am dark cycle, then at noon on the following day (i.e. the day on which the vaginal plug is found) the embryos are aged 'half-day postcoitum' or '0.5 d.p.c.'.

By 24 hours after fertilization, the embryo is still at the two-cell stage, and will continue to divide slowly without any increase in mass as it moves along the oviduct into the uterus for implantation 4.5 days after fertilization. The implanting embryo, called blastocyst, generates the first two cell lineages: the trophoectoderm and the inner cell mass. Once implantation has been achieved there is a dramatic increase in the growth rate of the embryo, particularly in the small group of pluripotential cells known as the primitive ectoderm, from which the fetus will develop. Between the 5th and 10th day after fertilization the basic body plan of the mouse is established. To summarize briefly, the mesoderm is formed and divided up into reiterated pairs of somite blocks, generating a segmented pattern along the anterior-posterior body axis. The neural plate is induced and folds up into the neural tube, and the placodes of the nose, ear, and eyes are formed. The neural crest cells start their migration, and the heart and circulatory system and limb buds are established. Therefore, it is during this period that many of the genes controlling the differentiation and morphogenesis of the adult organs are gradually brought into play.

The gestation period for the mouse embryo is 19-20 days, depending on the strain. The timing of the different stages is shown in **Table 1.1**, which is based on the development of F1 hybrids between C57BL/6 females and CBA males (adapted from Hogan *et al.*, 1994).

Table 1.1 Development of the mouse embryo

Age (days p.c.)	Features
0-1	one-cell egg
1	two-cell egg
2	morula, 4-16 cells
3	morula-blastocyst; trophectoderm formed
4	free blastocyst without zona
4.5	implanting blastocyst; primitive ectoderm
5	egg cylinder
6	proamniotic cavity in primitive ectoderm
6.5	embryonic axis determined
7	early to mid-primitive streak; amnion forming
7.5	late primitive streak; allantois appearing
8	1-7 somites; allantois contacts chorion
8.5	8-12 somites; turning of embryo
9	13-20 somites; heart begins to beat
9.5	21-29 somites; forelimb bud at level of somites 8-12
10	30-34 somites; hindlimb bud at level of somites 23-28
10.5	35-39 somites; tail rudiment
11	40-44 somites; spleen primordium
11.5	6- to 7-mm length, forefoot plate
12	7- to 9-mm, hindfoot plate
13	9- to 10-mm, whisker rudiments
14	11-12 mm
15	12-14 mm
16	14-17 mm
17	17-20 mm
18	19.5 - 22.5 mm
19	23 -27 mm; birth

The development of mammalian embryo requires the coordinated control of both cell proliferation and differentiation. The regional specification of cell growth and differentiation in early embryo depends on a continuous series of cell-to-cell interactions triggering specific developmental decisions. Since cytokines are recognized as the central participants in all cellular communication events, precise embryogenesis is essentially controlled by complex and finely balanced cytokine networks.

1.1.3 An overview of cytokines

Cytokines are a heterogeneous group of polypeptide mediators that have been associated with intercellular communication. They serve to coordinate all the important biological processes during embryonic development and in later life, including cell proliferation, differentiation and immune responses. These soluble molecules are produced by many cell types in addition to those of the immune system. Most cytokines are multifunctional, thus making their nomenclature and classification difficult. Nonetheless, the identity and characteristics of individual cytokines were gradually clarified as a consequence of the developments in molecular biology. Within the last 5 years, there has been an explosion of information on cytokines and cytokine receptors. It is expected that an increasing number of related mediators will be continuously added to this category.

a. Classes of cytokines

Various terms have been introduced in attempts to order the enormous number of acronyms and titles applied to soluble signaling molecules secreted by cells. Thus, the terms 'lymphokine', 'monokine', 'cytokine' and 'interleukin' are widely used, but the term 'cytokine' has emerged as a generic term to denote this group of soluble proteins. This term is used in the present thesis and most of the literature. The term 'interleukin' is used in the naming of specific cytokines, such as interleukin-2.

Identification and characterization of the many cytokines now known has resulted in a variety of names and classifications. In the following section, the major groups of cytokines will be described briefly according to current nomenclature. Since most cytokines have more than one function, there is considerable overlap in the biological effects and mechanisms of action of cytokines currently placed in different groups.

i) Growth factors

It has been known for many years that a number of small polypeptides are able to promote growth and division of various cell types in tissue culture. These are collectively known as growth factors (Clemens, 1991). However, their names may not accurately indicate their specificity. In many cases cell differentiation rather than proliferation is the main consequence of treating a cell type with a growth factor.

Table 1.2 Properties of major growth factors

Cytokines		Principal sources	Principal effects	Ref.
Epidermal growth factor	EGF	Many cell types	Proliferation of epithelial cells, bone resorption	Das *et al.*, 1992
Platelet-derived growth factor	PDGF	Megakaryocytes, monocytes, others	Chronic inflammation, tissue repair and wound healing	Rose *et al.*, 86 Deuel & Kawahara, 92
Fibroblast growth factor	FGF	Neuroectoderm, others	Proliferation of neuroectodermal cells, endothelial cell, fibroblast, angiogenic activity	Gospodarowicz, 92
Insulin-like growth factor	IGF	Liver, neural tissues	Growth of cartilage, mammary gland, stimulation of glucose uptake and protein synthesis	Hamblin, 93
Nerve growth factor	NGF	Neural tissue, salivary gland	Differentiation and survival of neural tissues	Hamblin, 93

Transforming growth factor (TGF) α and β were originally discovered in certain tumors which allow normal cell types to grow into malignant ('transformed') cells (Abbas *et al.*, 1991). However, it was an historical accident that linked these two factors together, and the names do not imply any structural or functional relationship. Indeed, their true physiological roles may be diametrically opposed since TGF-β is a growth *inhibitor* rather than a mitogen for most cell types (Clemens, 1991).

Table 1.3 Properties of transforming growth factors (TGFs)

Cytokines	Principal sources	Principal effects	Ref..
TGF-α	Tumor cells, keratinocytes, macrophages	EGF-like	Clemens, 93
TGF-β	Macrophages, T cells, chondrocytes	Growth inhibition of transformed cells, anti-inflammation, immunosuppression, wound healing	Hamblin, 93 Kuby, 94

ii) Interleukins

The term 'interleukin' was coined in the hope that by numbering these mediators (interleukin-1, interleukin-2, etc.), a universal nomenclature could be created. This can avoid the use of a 'function-based' name which could be misleading in terms of the overall activity of that molecule. However, the term 'interleukin' may prove unfortunate since it implies that these mediators only act as signals between leucocytes. Although many of them do have this function, most also have functions which bypass leucocytes entirely (Roitt *et al.*, 1993).

Up to now, a total of at least 15 cytokines have been proposed for the interleukin designation with official blessing. Generally, one or two new interleukins are added to the list each year (Janeway and Travers, 1994).

Table 1.4 Properties of interleukins (ILs)

(Aggarwal and Pocsik, 1992; Kuby, 1994; Janeway and Travers, 1994)

Cytokines	Principal sources	Principal effects
IL-1α, IL-1β	Macrophages, endothelial cells	Activation of B, T cells, macrophages Fever and inflammatory response
IL-2	T cells	T cell proliferation, stimulation of cytokine synthesis by T cells, activation of macrophages
IL-3	T cells	Synergistic action in hematopoiesis
IL-4	T cells	B cell activation, Ig E switch
IL-5	T cells	Growth and differentiation of eosinophil
IL-6	T cells, macrophages	Growth and differentiation of T and B cells, acute phase reaction, hematopoiesis
IL-7	Stromal cells	Growth of pre-B and pre-T cells
IL-8	Macrophages	Chemotactic for neutrophils, T cells
IL-9	T cells	Mast cell enhancing activity
IL-10	T cells, macrophages	Inhibition of cytokine synthesis
IL-11	Stromal cells, fibroblast	Synergistic action with IL-3 and IL-4 in hematopoiesis
IL-12	B cells, macrophages	Activation of natural killer cells
IL-13	T cells	B cell growth and differentiation, inhibits macrophage inflammatory cytokine production
IL-14	T cells, B cells	B cell proliferation
IL-15	T cells	IL-2 like

iii) Colony-stimulating factors

Colony stimulating factors are defined as proteins which stimulate the clonal growth of bone-marrow cells *in vitro* (Abbas *et al.*, 1991; Clemens, 1991). Although only an operational definition, the name colony stimulating factor (CSF) is conceptually useful since this group of cytokines function mainly to regulate proliferation and differentiation of various types of hematopoietic cell, such as granulocytes, macrophages and erythroid cells. There is functional overlap between CSFs and some of the interleukins described earlier. For example, IL-3 was formerly called multi-CSF because it promotes growth and differentiation of both myeloid and erythroid cell precursors. Thus its activities overlap with those of granulocyte/macrophage-CSF.

Table 1.5 Properties of major colony stimulating factors
(Abbas *et al.*, 1991; Clemens, 1991; Aggarwal and Pocsik, 1992)

Cytokines		**Principal sources**	**Principal effects**
Granulocyte-Macrophage colony stimulating factor	GM-CSF	Macrophage, T cells, endothelial cells, fibroblasts	Proliferation of granulocyte and macrophage precursors
Granulocyte colony stimulating factor	G-CSF	Macrophages endothelial cells, fibroblasts	Proliferation of granulocyte progenitors
Macrophage colony stimulating factor	M-CSF	Monocytes endothelial cells, fibroblasts	Proliferation of macrophage precursors

iv) Interferons

Interferons (IFNs) were originally identified as agents produced by virus-infected cells which can protect cells against further viral infections. Hence, the name was derived from viral 'interference' (Clemens, 1991). It is now clear, however, that IFNs can elicit many other changes in cellular behavior, including effects on cell growth and differentiation and modulation of the immune system. The multiple species of IFNs are now classified into three groups, α, β and γ, on the basis of their distinct antigenic properties. Whilst there is only one IFN-γ and one IFN-β gene, there are at least 23 different genetic loci for the α interferons of which 15 correspond to functional genes (Hamblin, 1993).

IFN-α is produced by leucocytes while IFN-β is produced by fibroblasts, in response to viral infection and stimulation with natural or synthetic double stranded RNAs. They have potent antiviral activity but at higher concentration have antiproliferative activity against both normal and tumor cells. They can enhance the expression of Class I MHC gene products and enhance natural killer (NK) cell activity and thus playing a role in immunoregulation (Hamblin, 1993).

IFN-γ has potent immunoregulatory effects on a variety of cells including activation of macrophages, enhanced production of IgG by B lymphocytes and increased expression of Classes I and II MHC gene products. IFN-γ counteracts the effect of IL-4 on B cells and thus inhibiting the activity of T helper cells (Mosmann and Coffman, 1989).

Table 1.6 Properties of interferons (IFNs)

(Clemens, 1991; Kuby, 1994; Aggarwal and Pocsik, 1992; Janeway and Travers, 1994)

Cytokines	Principal sources	Principal effects
IFN-α	T and B cells, monocytes	Anti-viral effect, increase MHC Class I expression
IFN-β	Fibroblasts, endothelial cells	
IFN-γ	T cells, natural killer cell	Macrophage activation, increase MHC expression, decrease cytokine synthesis

v) Tumor necrosis factor

As with many cytokines, the name 'tumor necrosis factors' (TNFs) reflects the history of these agents, and is misleading because although TNFs can induce regression of some tumors, it gives no clue to their many other actions (Clemens, 1991). There are two species of TNF, α and β, that show considerable homology in their amino acid sequences. The major cellular source of TNF-α is from macrophages activated by agents such as bacterial lipopolysaccharide while TNF-β is produced mainly by activated T lymphocytes (Kuby, 1994).

In general, production of the TNFs forms part of the inflammatory response to invasion by micro-organisms and is an important part of host defenses against infection. In addition, TNFs can enhance the activity of granulocytes and eosinophils,

leading to increased phagocytosis and other mechanisms of cell killing. These effects undoubtedly contribute to the anti-tumor actions of the TNFs *in vivo*. However, TNFs also have a direct cytostatic or cytotoxic effect on some tumor cells, demonstrable *in vitro* (Clemens, 1991).

Table 1.7 Properties of tumor necrosis factors (TNFs)
(Clemens, 1991; Hamblin, 1993; Kuby, 1994)

Cytokines	Principal sources	Principal effects
TNF-α	Macrophages, natural killer cells	cytotoxic to tumor cells, Inflammatory responses, activation of granulocytes, eosinophils, macrophages
TNF-β	T cells	

b. Cytokine networks

An important aspect of cytokine activity is that they interact with each other in a variety of networks *in vivo*. The following properties of cytokines attribute to the formation of networks:

1. Many individual cytokines are produced by different cell types
2. Most cytokines have several functions, i.e., pleiotropic

3. Cytokine actions are often shared by several different cytokines, i.e., redundant
4. Cytokines often influence the synthesis of other cytokines, leading to a cascade of biological effects
5. Cytokines often influence the action of other cytokines, either antagonistically or synergistically

Cytokines, like other polypeptide hormones, initiate their action by binding to specific receptors on the surface of target cells. Sharing similar or overlapping biological effects by several cytokines is probably due to use of common receptors on the target cells or to similarities in the intracellular pathways activated by different receptors. The spectrum of effects brought about by a given cytokine can depend both on the nature and on the functional state of the target cell. The latter can of course be modulated by other influences (including other cytokines) which may regulate receptor activity and/or post-receptor pathways inside the cell (Clemens, 1991).

c. Role of cytokines in the whole organism

As cytokines are signaling molecules that mediate cell-to-cell interactions, it may be reasonable to think that cytokines have important roles in growth, development, and maintenance of advanced multicellular, multi-organ species (e.g. mammals) where the ordered and unharmed assembling and functioning of cells are vital to normal morphogenesis, not only during embryonic and neonatal development but also through adolescence and adulthood (Meager, 1990).

All exogenous stimuli cause localized perturbations in cells, resulting in imbalances in cellular metabolism. In most circumstances, cells will respond rapidly to counteract any subsequent imbalances by internal adjustment of metabolic processes and by the secretion of mediators to communicate the nature of the imbalance to other cells and stimulate such cells to respond accordingly. Therefore, the balanced production and actions of cytokines are essential in maintaining an organism in good condition.

1.1.4 Cytokine and receptor gene expression in mouse embryonic development

During embryogenesis, the ordered proliferation of cells and their differentiation to form specialized organs and tissues is essential. This process of development must be largely dependent upon and controlled, both temporally and spatially, by the episodic and often cyclical release of many different cytokines and their subsequent concerted actions (Jessell and Melton, 1992). The roles that cytokines and their receptors play in murine development is at present largely circumstantial and unexplored. Nonetheless, knowledge about the changes in gene expression is essential to understanding mammalian development.

Embryonic development can be roughly splitted into two phases which are separated at the time of implantation. Most of the analysis of differentially expressed genes were done with early mouse embryo during preimplantation.

a. Murine embryonic stem cell model

The preimplantation mouse embryo develops from a fertilized egg into a hatched blastocyst of about 100 cells over a 5-day period. The major problem in determining cytokine expression pattern during this period is the difficulty of obtaining sufficient quantities of timed embryos for experimentation. As an alternative approach, many studies have been performed on embryonal carcinoma (EC) cells or embryonic stem (ES) cells in an attempt to determine which factors might be expressed by cells of

the early embryo and what their function might be in regulating differentiation. EC cells from different teratocarcinomas have a variety of properties in common with the inner cell mass (ICM) of the blastocysts (Mummery and Eijnden-van-Raaij, 1990). ES cells, on the other hand, are pluripotential cells directly isolated from ICM of cultured mouse blastocysts (Evans and Kaufman, 1981). They have attracted much interest, both as a means of studying the molecular basis of early mouse development and as a route to genetically manipulating the genome of mice (Bradley *et al.*, 1992; Koller and Smithies, 1992).

b. Leukemia Inhibitory Factor (LIF) in mouse embryos

Characterization of the expression and function of LIF in cultured ES cells has implicated this factor as a regulator of differentiation of the pluripotential stem cell in the early mouse embryo (Williams *et al.*, 1988; Smith *et al.*, 1988). LIF transcripts are found in preimplantation blastocysts (3.5 d.p.c.), extraembryonic tissue of 7.5 d.p.c. and in the placenta of 9.5, 10.5 and 12.5 d.p.c. embryos (Conquet and Brulet, 1990). Smith *et al.* (1992) detected the transcripts are significantly down-regulated by the onset of gastrulation at 7.5 days postcoitum. The studies by Stewart *et al.* (1992) revealed that LIF is essential for the implantation of embryo. Female mice lacking a functional LIF gene by targeted mutagenesis are fertile, but their blastocysts fail to implant and do not develop. LIF regulates implantation by priming the uterus as well as regulating cell proliferation in the blastocyst. There is a coincidence in timing existed between arrival of the embryo in the uterine lumen and the onset of LIF expression, as delineated in

Figure 1.1. On the 5th day following implantation and decidua formation, the endometrial glands (site of LIF expression) degenerate and stop expressing LIF (Bhatt *et al.*, 1991; Stewart, 1994).

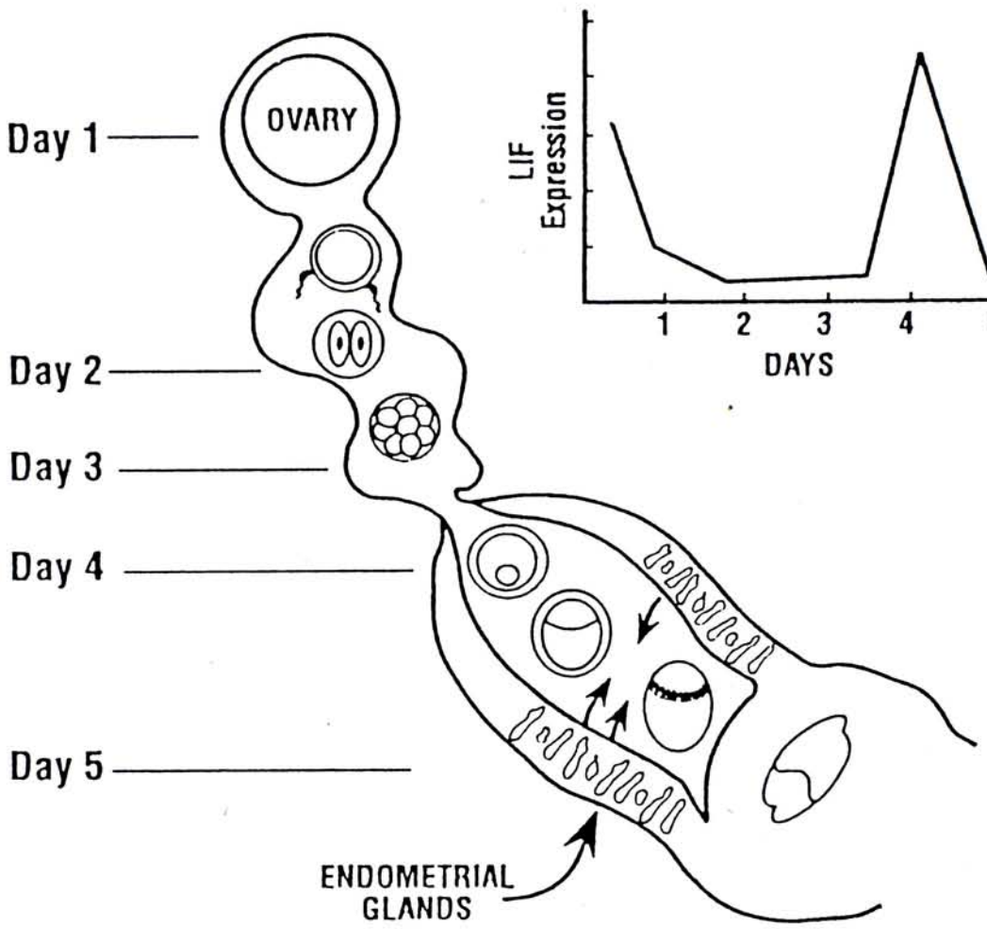


Figure 1.1 Diagram summarizing the pattern of LIF expression in the uterus during the first 5 days of pregnancy. The stages of embryonic development are shown with LIF expression from endometrial glands occurring on the 4th-5th days. By the 5th day, implantation has occurred (Stewart, 1994).

c. IL-6 in mouse embryo

IL-6 shares a number of biological properties with LIF. Murray *et al.* (1990) detected the presence of both IL-6 and LIF mRNAs in mouse blastocyst at 3.5 days of gestation. It is therefore believed that IL-6 also takes part in fetal development, perhaps in regulating differentiation and proliferation of uncommitted cells of the inner cell mass or in the development of the first hematopoietic stem cells (Lee, 1992).

d. Ciliary Neurotrophic Factor (CNTF) in mouse embryo

CNTF is a polypeptide that promotes the survival and/or differentiation of a variety of neuronal cell types including ciliary, sensory and sympathetic and motor neurons *in vitro* (Sendtner *et al.*, 1994). Stewart and co-workers (1994) have been unable to detect CNTF transcripts in the mouse uterus or pre-implantation embryo. Homozygous mice deficient in CNTF production were derived and were overtly normal during the first postnatal weeks. But with increasing age, spinal motor neurons exhibited progressive degeneration which was functionally reflected by a significant reduction in muscle strength (Masu *et al.*, 1993). This result has shown that elimination of CNTF gene did not affect the number of motor neurons during embryonic development but it has an essential maintenance function for motor neurons in the postnatal period. It seems the role of CNTF in embryogenesis remains elusive, however, CNTF is also able to maintain the pluripotentiality of ES cells (Conover *et al.*, 1993).

e. TNF-α and TNF-β in mouse embryos

These cytokines act as mediators of inflammatory response, programmed cell death and immune cell growth and differentiation. Kohchi and co-workers (1994) demonstrated that both TNF-α and TNF-β were expressed constitutively in almost all organs of fetus as well as the placenta from 10th day of gestation onwards. It was then hypothesized that the major roles of TNFs in embryonic development are their involvement in programmed cell death, in the regulation of cellular growth and differentiation, and in the remodeling of extracellular matrix (Wride and Sanders, 1995).

f. TGF-α in mouse embryos

Maternal transcript of TGF-α apparently disappeared and were resynthesized in zygote to blastocyst stage (Rappolee *et al.*, 1988). TGF-α is able to bind efficiently to the epidermal growth factor (EGF) receptor. Since both their mRNA and proteins are detectable in mid-gestation mouse embryos, TGF-α is proposed to be the primary component of embryonic EGF activity (Popliker *et al.*, 1987; Twardzik, 1985).

g. TGF-β in mouse embryos

TGF-β is a group of multi-functional regulatory polypeptides that are secreted by a variety of cells and play a crucial role in cell proliferation, differentiation, migration and extracellular matrix production (Roberts and Sporn, 1990). Rappolee *et al.* (1988) detected that the transcription of TGF-β1 started from fertilization and

increased continuously up to the blastocyst stage. TGF-β1 protein has also been detected from day 9 onwards in post-implantation embryos (Heine *et al.*, 1987). More recently, Kohchi *et al.* (1994) detected constitutive expression of TGF-β in various fetus organs and placenta from 10th day on throughout development, like TNF-α and TNF-β. Based on its activities and expression during mouse embryogenesis, TGF-β is widely assumed to have important functions in mammalian development.

h. Stem cell factor / c-kit

The recently described stem cell factor (SCF) (Zsebo *et al.*, 1990) is the ligand binding to *c-kit* (Chabot *et al.*, 1988), a cell surface tyrosine kinase receptor. Evidence has been obtained that this SCF ligand/*c-kit* receptor pair is essential for hematopoiesis (Migliaccio *et al.*, 1991; Ogawa *et al.*, 1991).

Following the discovery of the hematopoietic growth factor SCF, Matsui *et al.* (1990) studied the embryonic expression and reported that the transcript of SCF was found in mouse embryo aged from 10.5 to 17.5 d.p.c.. *In situ* hybridization study clearly revealed that the SCF gene was expressed in tissues associated with the migration, proliferation and differentiation of hematopoietic stem cells, presumptive melanoblasts and primordial germ cells, but were also present in a variety of other sites (Matsui *et al.*, 1990).

Palacios and Nishikawa (1992) have studied the developmentally regulated expression and function of *c-kit* receptor in mouse embryo using immunofluorescence staining method. The findings indicate that within the hematopoietic system of the developing embryo, cells bearing *c-kit* receptors are already present in the yolk sac at day 8 to 8.5 (before fetal blood circulation has started). *c-kit*$^+$ hematopoietic cells are then found in the liver from day 10 and in the thymus from day 11 of gestation. In these organs, *c-kit*$^+$ cells expand up to day 15 to 16 and thereafter switch off expression of *c-kit*. By day 17, only few hematopoietic cells are still *c-kit*$^+$ in these organs. In the same study, the participation of SCF/*c-kit* system during lymphocyte development in mouse embryo was also demonstrated. SCF/*c-kit* contribute to this process by supporting cell survival and enhancing the response of the developing lymphoid precursor to other cytokines (e.g. IL-2, IL-3, IL-4, IL-7).

i. Other cytokines in mouse embryos

Hematopoietic development

Many cytokines have been shown to be important to hematopoietic development, such as erythropoietin (Epo), G-CSF, M-CSF, GM-CSF, IL-1, IL-3, IL-4 and IL-6 (Jessell and Melton, 1992). SCF and its receptor, *c-kit*, are also critical to hematopoiesis. Using murine embryonic stem (ES) cell *in vitro* system, Schmitt and co-workers (1991) demonstrated that Epo, M-CSF, IL-4, IL-6, SCF and *c-kit* were expressed during the first 24 days of ES cell differentiation. The expression of these cytokines reflects their importance in hematopoietic development. SCF and its receptor

(*c-kit*) have been reported to have IL-3 like activities on hematopoietic colony forming units (Anderson *et al.*, 1990) while IL-4 in combination with IL-6 stimulates colony formation of various hematopoietic progenitor cells (Rennick *et al.*, 1989). Thus it is conceivable that the expressed cytokines have important synergistic effects on very primitive hematopoietic cells as well as having crucial roles early in fetal development. In contrast, neither IL-3 nor GM-CSF transcripts were detected in the same experiment by Schmitt *et al.* (1991). These two factors are well documented to have effects on immature myeloid cells, however, the lack of transcription during the *in vitro* hematopoietic development of ES cells suggests that they are not critical to this process.

The *c-fms* gene encodes the receptor for the M-CSF (Sherr *et al.*, 1985). The interaction of M-CSF with its receptor, *c-fms*, is required for the differentiation of cells in monocyte-macrophage lineage in the hematopoietic system. Regenstreif and Rossant (1989) utilized *in situ* hybridization techniques to determine the expression of *c-fms* and M-CSF during mouse embryogenesis. High levels of *c-fms* and M-CSF were detected in maternal tissue starting from 7.5 days of gestation. Their transcripts were observed in embryonic tissues only from 9.5 days onwards. The time course and spatial pattern of expression of these two genes suggest a functional role for the *c-fms* receptor and its ligand, M-CSF, in embryonic development.

The insulin-like growth factors (IGFs) are considered primarily as fetal mitogen involved in the development of embryonal tissues (Haselbacher *et al.*, 1985). IGF-I mRNA transcripts are detected at every stage from the fully grown, meiotically

competent oocyte to the blastocyst stages of development. The expression pattern changed dramatically in which the level declined from the oocyte to 8-cell embryo and then increased from the 8-cell to blastocyst stages (Doherty *et al.*, 1994).

j. Cytokine receptors

Cytokine molecules mediate their biological function by binding to specific receptors expressed on target cells. The evidence that cytokines are present in embryos has now become unquestionable. The effects of such molecules could be regulated by their abundance and tissue specificity during development or by alterations in the expression of either the receptors to which they bind or other elements in the signaling pathway that they induce (Mummery and van den Eijnden-van Raaij, 1990). From the *in vitro* studies, there are clear indication that the expression of cytokine receptors is indeed modulated during differentiation and for at least some of these there is differential expression in embryos themselves. For example, Schmitt and co-workers (1991), using murine ES cell system, detected the expression of IL-1R type I, IL-3R, IL-4R, IL-6R, EpoR, G-CSFR and *c-fms* in differentiated ES cells and their levels increased throughout the time course of the experiment. IL-3 expression was not observed at any time during ES cell development, though its receptor was detected as early as day 4 of ES cell differentiation. In most cases, the receptor genes were transcribed before the corresponding cytokines genes (Schmitt *et al.*, 1991). In another study by Mummery and Eijnden-van Raaij (1990), receptors for TGF-β was found in both undifferentiated and differentiated ES cells. Other studies were also carried out

for other cytokine receptors, like IGF-II receptors were detected on blastocysts and both IGF-I and IGF-II receptors in membrane preparations of day 9-12 gestation mouse embryos (Smith *et al.*, 1987). EGF receptors were found present on embryonic and extraembryonic tissues. The earliest time of detection was on giant trophoblast cells of a 5-day blastocyst grown for 2-3 days in culture (Adamson and Meek, 1984).

There is an emerging body of literature in which cytokines or their genes were detected during murine embryogenesis. The observations of the studies were summarized in **Table 1.8**.

Table 1.8 Summary of cytokine gene expression in mouse embryos.

Factors	Occurrence	Reference
IL-1α	• Absent in mid-late embryos (10-17 d.p.c.)	Kohchi *et al.*, 1994
IL-1β	• Blastocyst stage	Rothstein *et al.*, 1992
	• Absent in mid-late embryos (10-17 d.p.c.)	Kohchi *et al.*, 1994
IL-3	• Absent in 3.5 d.p.c. blastocyst	Murray *et al.*, 1990
	• Absent in mid-late embryos (10-17 d.p.c.)	Kohchi *et al.*, 1994
IL-6	• 8-cell stage and blastocyst	Rothstein *et al.*, 1992
	• 3.5 d.p.c. Blastocysts	Murray *et al.*, 1990
	• Absent in mid-late embryos (10-17 d.p.c.)	Kohchi *et al.*, 1994
LIF	• 3.5 d.p.c. Blastocysts	Conquet and Brulet, 1990 Murray *et al.*, 1990
	• Uterus during the first 5 days of pregnancy	Bhatt *et al.*, 1988
	• Extraembryonic ectoderm of 7.5 d.p.c. embryos and 9.5, 10.5, 12.5 d.p.c. placentas	Conquet and Brulet, 1990
	• Absent in mid-late embryos (10-17 d.p.c.)	Kohchi *et al.*, 1994
GM-CSF	• Absent in 3.5 d.p.c. blastocysts	Murray *et al.*, 1990
G-CSF	• Absent in 3.5 d.p.c blastocyst	Rappolee *et al.*, 1988
	• Absent in mid-late embryos (10-17 d.p.c.)	Kohchi *et al.*, 1994
M-CSF & *c-fms*	• In maternal tissue from 7.5 d.p.c. onwards In embryonic tissue starting from 9.5 d.p.c.	Regenstreif and Rossant, 1989
IGF-I	• Oocyte to blastocyst	Doherty *et al.*, 1994
EGF	• Absent in 3.5 d.p.c blastocyst	Rappolee *et al.*, 1988
NGF-β	• Absent in 3.5 d.p.c blastocyst	Rappolee *et al.*, 1988
bFGF	• Absent in 3.5 d.p.c blastocyst	Rappolee *et al.*, 1988
PDGF-A	• 3.5 d.p.c. blastocyst	Rappolee *et al.*, 1988

Table 1.8 (continued)

Factors	Occurrence	Reference
TGF-α	• 3.5 d.p.c. blastocyst	Rappolee *et al.*, 1988
	• 7 d.p.c. embryos and onwards	Popliker *et al.*, 1987
TGF-β	• 3.5 d.p.c. blastocyst	Rappolee *et al.*, 1988
	• 9 d.p.c. embryos and onwards	Heine *et al.*, 1987
	• Placentas (10-17 d.p.c.) All embryo organs (10 d.p.c. and onwards)	Kohchi *et al.*, 1994
TNF-α	• Placentas (10-17 d.p.c.) All embryo organs (10 d.p.c. and onwards)	Kohchi *et al.*, 1994
TNF-β	• Placentas (10-17 d.p.c.) All embryo organs (10 d.p.c. and onwards)	Kohchi *et al.*, 1994
SCF	• 10.5-17.5 d.p.c.	Matsui *et al.*, 1990
c-kit	• Increases from 8 d.p.c. to 15 d.p.c. and decreases thereafter	Palacois and Nishikawa, 1992
IFN-γ	• Blastocyst stage	Rothstein *et al.*, 1992
	• Absent in mid-late embryos (10-17 d.p.c.)	Kohchi *et al.*, 1994
CNTF	• Absent in uterus or preimplantation embryo	Stewart, 1994

1.2 Neuropoietic cytokines

A number of cytokines sharing limited sequence homology have been grouped as a family because of partially overlapping biological activities, receptor subunit promiscuity, and the prediction of a shared secondary structure. Since several of these cytokines regulate gene expression and cell number in the nervous and hematopoietic systems, this specific group is termed the neuropoietic cytokine family (Fann and Patterson, 1994). This unusual family of cytokine could mediate 'cross-talk' between the nervous and immune systems, in both directions.

1.2.1 Family members

Inaugural members of the neuropoietic family include leukemia inhibitory factor (LIF), interleukin-6 (IL-6), oncostatin M (OSM), ciliary neurotrophic factor (CNTF) and interleukin-11 (IL-11) (Patterson, 1994; Tadamitsu, 1994).

Although they share only very limited sequence homology, they exert very similar effects on a variety of tissues. Several of these proteins can, for instance, induce the same set of acute-phase response proteins in liver, support the self renewal of cultured embryonic stem cells, inhibit lipogenesis, and enhance the survival of cultured motor neurons. Despite their limited homology, the neuropoietic cytokines are predicted to share a four antiparallel helix bundle secondary structure. These ligands of

very similar structure bind to receptor subunits that share various degrees of homology. Subsequent studies on the receptors have revealed not only structural homology among receptors but the sharing of a common receptor transducing subunit (gp130) and similar signal-transduction pathways (Fann and Patterson, 1994).

1.2.2 Shared signal transducer gp130

The overlaps in receptors and signal cascades offer a convenient molecular explanation for the often redundant biological activities of the neuropoietic cytokines. IL-6, LIF, OSM, CNTF and IL-11 receptor systems are known to utilize a common signal transducer, gp130. Signals from these cytokines are believed to be initiated by the ligand-induced homo- or heterodimerization of gp130, which leads to the interaction of their cytoplasmic regions, resulting in activation of associated tyrosine kinase(s). The receptor binding complexes for the family members are schematically illustrated in **Figure 1.2**.

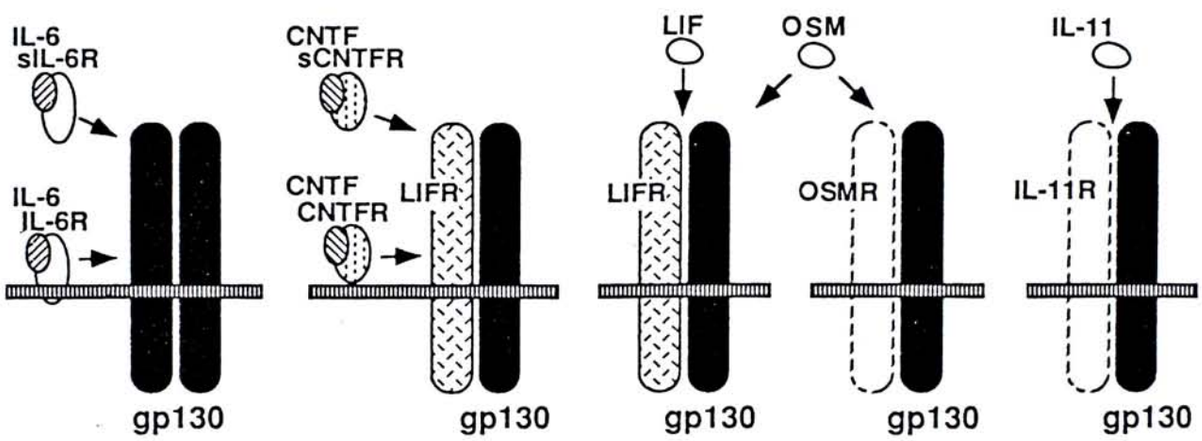


Figure 1.2 Common signal-transducing receptor components belonging to the neuropoietic cytokine family (adapted from Tadamitsu *et al.*, 1994).

1.2.3 LIF, CNTF and OSM inhibit differentiation of embryonic stem cells

In the case of LIF receptor (LIFR) system, the LIFR forms a one-to-one heterodimer with gp130 (**Figure 1.2**), and this combination generates a high affinity LIF-binding site. Interestingly, OSM also binds with LIFR-gp130 complex with high affinity in addition to its own receptor complex which consists of OSMR and gp130 (Gearing *et al.*, 1992). The high affinity form of CNTF receptor includes LIFR-gp130 plus a separate CNTFR subunit. The sharing of receptor by LIF, OSM and CNTF serves to explain the similar biological functions mediated by these cytokines. Among the multiple functions, maintenance of the undifferentiated state of embryonic stem cells play an important role in embryonic development.

LIF has long been documented for its ability to inhibit the differentiation of pluripotent embryonic stem cells *in vitro* (Williams *et al.*, 1988; Smith *et al.*, 1988). Later, CNTF (Conover *et al.*, 1993) and OSM (Rose *et al.*, 1994) were also demonstrated that they are similar to LIF in its ability to maintain the pluripotent phenotype of these cells. Since these cytokines have certain *in vitro* effect on embryonic development, gene targeting experiment by 'knock-out' mice were carried out to study their *in vivo* functions (Masu *et al.*, 1993; Stewart *et al.*, 1992). To achieve this, gene clones of the cytokines have to be ready first. The human and mouse clones for LIF (Gearing *et al.*, 1987; Gough *et al.*, 1988) and CNTF (Kaupmann *et al.*, 1991; Lam *et al.*, 1991) genes were subsequently obtained and sequenced. However, only human OSM gene was cloned and sequenced by Malik *et al.* in 1989. Although

murine OSM has been identified, it has not yet been cloned and analyzed up to the commencement of this project. Therefore, a preliminary study of murine OSM gene would be included in the present project.

1.3 Biology of Oncostatin M (OSM)

OSM was originally isolated from media conditioned by U937 human histiocytic leukemia cells induced to differentiate into macrophage-like cells by treatment with phorbol 12-myristate 13-acetate (PMA) (Zarling *et al.*, 1986). It was later determined as a growth regulatory molecule which could inhibit the growth of certain tumor cell lines and thus it won the name 'onco-' 'statin' (i.e. tumor stable).

1.3.1 Physical properties of OSM

OSM is found as a glycoprotein, with a Mr of 28,000 (Zarling *et al.*, 1986). This mature form of 196 amino acids is derived from a 252 amino acid precursor by the removal of a 25 amino acid N-terminal leader sequence and a 31 amino acid C-terminal peptide. There are two potential N-glycosylation sites together with sites for O-linked glycosylation (Bruce *et al.*, 1992). It is a very stable protein, retaining its biological activity in extremes of pH (2-11) and after heating (56°C, 1 hr).

The mature OSM is predicted to adopt a four alpha-helical bundle structure similar to that determined for growth hormone (Rose *et al.*, 1991). OSM contains five cysteine residues with four of them forming two intramolecular disulfide bonds. The second of these has been found to be essential of biological activity. Mutational analysis has shown that the amphiphilic C-terminal helix, and other discontinuous regions within the molecule, are involved in receptor binding (Kallestad *et al.*, 1991).

1.3.2 Biological activities of OSM

OSM acts to inhibit or promote various biological activities according to the cell type affected and they are summarized in **Table 1.9**. As with most cytokines, these activities are diverse and, in some cases, appear contradictory.

Table 1.9 Summary of *in vitro* effects of OSM (Bruce *et al.*, 1992).

Solid tumor cells
- Inhibits the growth of a solid tumor cells from a wide variety of sources.

Hematopoietic cells
- Induces the differentiation of human and murine leukemia cells.

Embryonic stem cells
- Blocks the differentiation of totipotent mouse embryonic stem cells.

AIDS-related Kaposi's sarcoma cells
- Is produced by AIDS-KS cells and acts in autocrine and paracrine fashion as a mitogen for these cells.
- Stimulates the production of IL-6 in these cells.

Hepatic cells
- Induces acute phase protein synthesis in HepG2 cells.

Fibroblasts
- Stimulates the growth of several fibroblast cell lines.

Endothelial cells
- Induces the production of IL-6 in human umbilical vein endothelial cells.
- Inhibits the growth of bovine aortic endothelial cells and stimulates the cells to produce plasminogen activator.

The range of *in vitro* biological activities presently attributed to OSM suggests its potential role in hematopoiesis, embryonic development, inflammation and pathogenesis of AIDS-related Kaposi's sarcoma.

1.3.3 Molecular aspect of OSM

Zarling's group first discovered OSM in 1986 and the human OSM was later cloned and sequenced by Malik *et al.* in 1989. The entire human gene spans approximately 5 kb, and the mRNA (about 2 kb) is composed of 3 exons. The first exon contains 5' non-coding region and the coding sequence of the first 10 amino acids of the signal peptide. The second exon encodes the remainder of the signal peptide and the first 34 residues of the mature molecule. The last exon encodes the remaining 193 amino acids and contains all of the 3' non-coding sequences present in the cDNA clone (Malik *et al.*, 1989). The human OSM cDNA and protein precursor were schematically represented in **Figure 1.3**. The 3' non-coding region contains several AU rich sections of the type that have been suggested to confer mRNA instability (Shaw and Kamen, 1986).

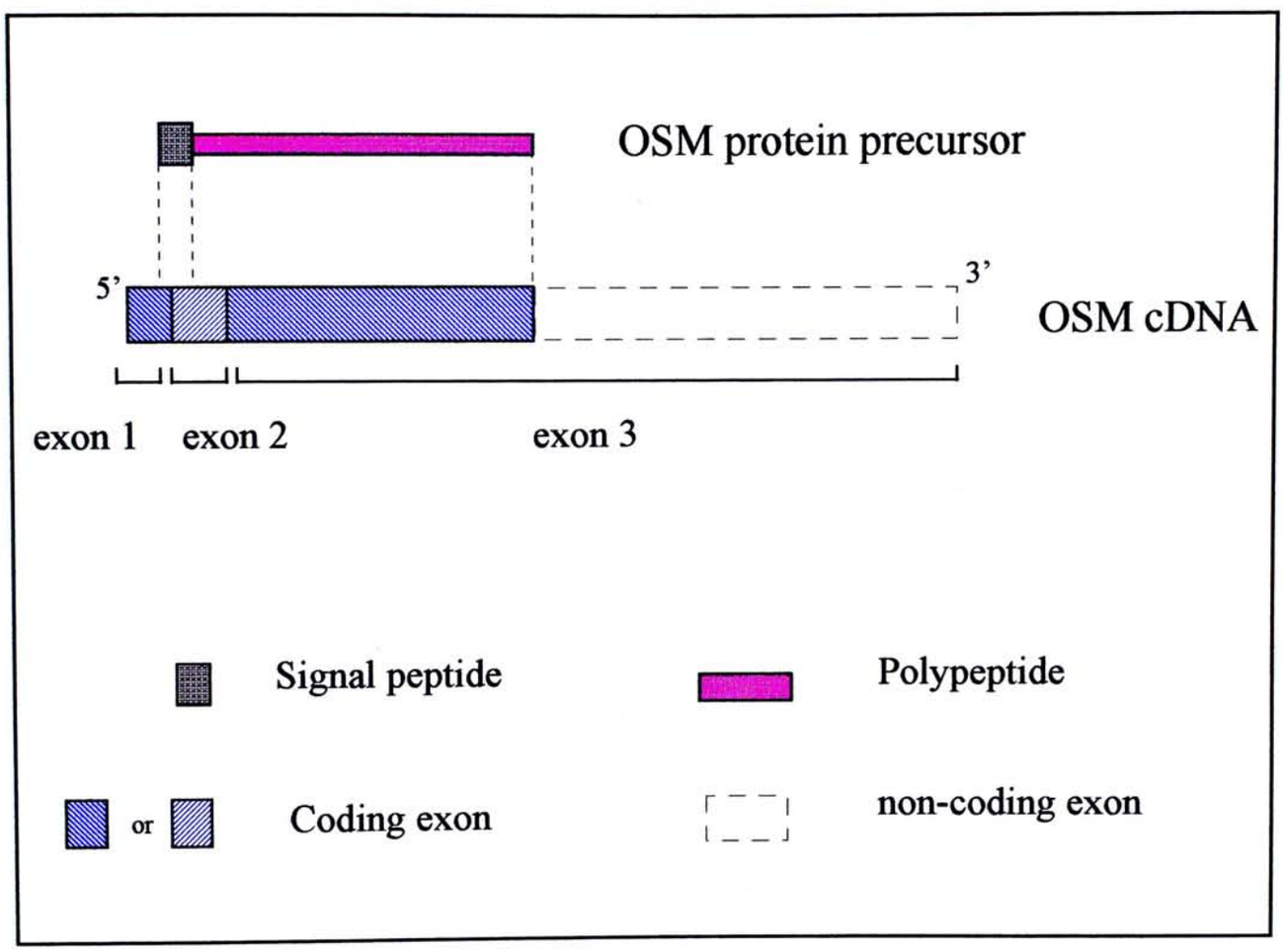


Figure 1.3 Schematic representation of the human OSM cDNA and protein precursors.

The gene for human OSM is located at 22q12 and lies within about 10 kb of the LIF gene (Rose *et al.*, 1993). The expression of OSM mRNA is generally restricted to hematopoietic cells including activated T cells and monocytes. The only known non-hematopoietic source of OSM RNA is cells derived from AIDS-related Kaposi's sarcoma (Miles *et al.*, 1992).

Although murine OSM has already been identified, its gene has not yet been cloned up to the day this project started. Therefore, a preliminary study on mouse OSM gene was carried out in the present study.

1.4 Aims of the study

Cytokines mediate cell-to-cell communication and bring about subsequent reactions. During development, cytokines may act as direct intercellular signals by regulating proliferation and/or differentiation of competent target cells or as indirect signals by inducing the expression of a cascade of other genes which in their turn affect specific developmental events. However, the function of cytokines in embryogenesis remains an intriguing problem.

To understand better the role of cytokines and their receptors during the embryonic development, we investigated the expression of a number of genes. Expression studies were accomplished by using reverse transcription-polymerase chain reaction (RT-PCR) to determine the relative mRNA levels throughout the developmental time course (7.5 d.p.c. to 17.5 d.p.c.). The time point at which a particular factor and its receptor undergoes significant expression will suggest when that factor begins to play a role in the differentiation process. Likewise, the lack of expression of a particular cytokine and its receptor would be strong evidence that the factor is not critical for development at that stage.

In the second part of the study, our focus turned to a novel cytokine, Oncostatin M (OSM), which belongs to the growing neuropoietic family. OSM, like LIF and CNTF, is demonstrated to inhibit differentiation of pluripotential embryonic stem cells. This property implicates its role in development. Human OSM gene has already been cloned and sequenced for a long time but, interestingly, the mouse gene was not studied

up to date. In the present project, a preliminary molecular analysis on mouse OSM gene was carried out. The PCR-cloned human OSM gene fragments were used as probe to hybridize with mouse genome.

Hopefully, the expression pattern of various cytokines during mouse embryonic development will accelerate the understanding of mammalian embryogenesis and speeding the benefits of embryo biotechnology to human health.

Chapter 2

Cytokine gene expression during mouse embryonic development

2.1 Introduction

2.1.1 Rationale

The aim of this part of project was to determine the expression profile of various cytokines during mouse embryonic development using Reverse Transcription-Polymerase Chain Reaction (RT-PCR). This method allows the detection and semi-quantitative assessment of changes in mRNA level of various genes at different stages of development.

Total RNA of five different time-pointed mouse embryos, ranging from 7.5 d.p.c. to 17.5 d.p.c., were extracted for analysis. Entire population of mRNA molecules was first converted into cDNA by priming with Oligo d(T) in reverse transcription. For significant comparisons of the small amounts of cytokine mRNAs present in embryos, cDNA samples were first normalized by PCR using primers specific for the constitutively expressed glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene. The normalization allowed the use of equivalent amounts of cDNA for each assay.

The analysis procedures following normalization are schematically depicted in **Figure 2.1**. To detect expression levels in 5 different time points of embryo, specific primers designed for over 30 mouse cytokine and receptor genes were initially used to perform 50 cycle-PCR upon normalized RT samples. Intensity of the resulted DNA bands as visualized in agarose gel provided a means of comparison of mRNA quantity among the 5 developmental stages. According to the results, cytokines and receptors were then grouped into '*no expression*', '*consistent*' and '*regulatory*' types correspondingly. To achieve semi-quantitative assessment, cycle number of PCR should lie on the exponential phase of amplification. Therefore, cycle number for '*consistent*' group were further lowered down to 30.

Southern hybridization was used to prove that '*no expression*' cytokines was due to the absence of their mRNA from mouse embryos at selected stages but not due to any problem occurred in PCR. On the other hand, cycle titration of PCR, dot blotting and subsequent hybridization were done for all genes having consistent or regulatory levels as observed at 30- or 50-cycle PCR. These steps could achieve the following goals:

1) validation of amplified sequences by hybridizing with internal probes
2) more precise quantitative comparison of gene expression level by densitometric measurement of the hybridization signals

The appropriate amplification cycle schedule for each gene was determined empirically to allow a minimal yet detectable signal, lying on the exponential phase of PCR.

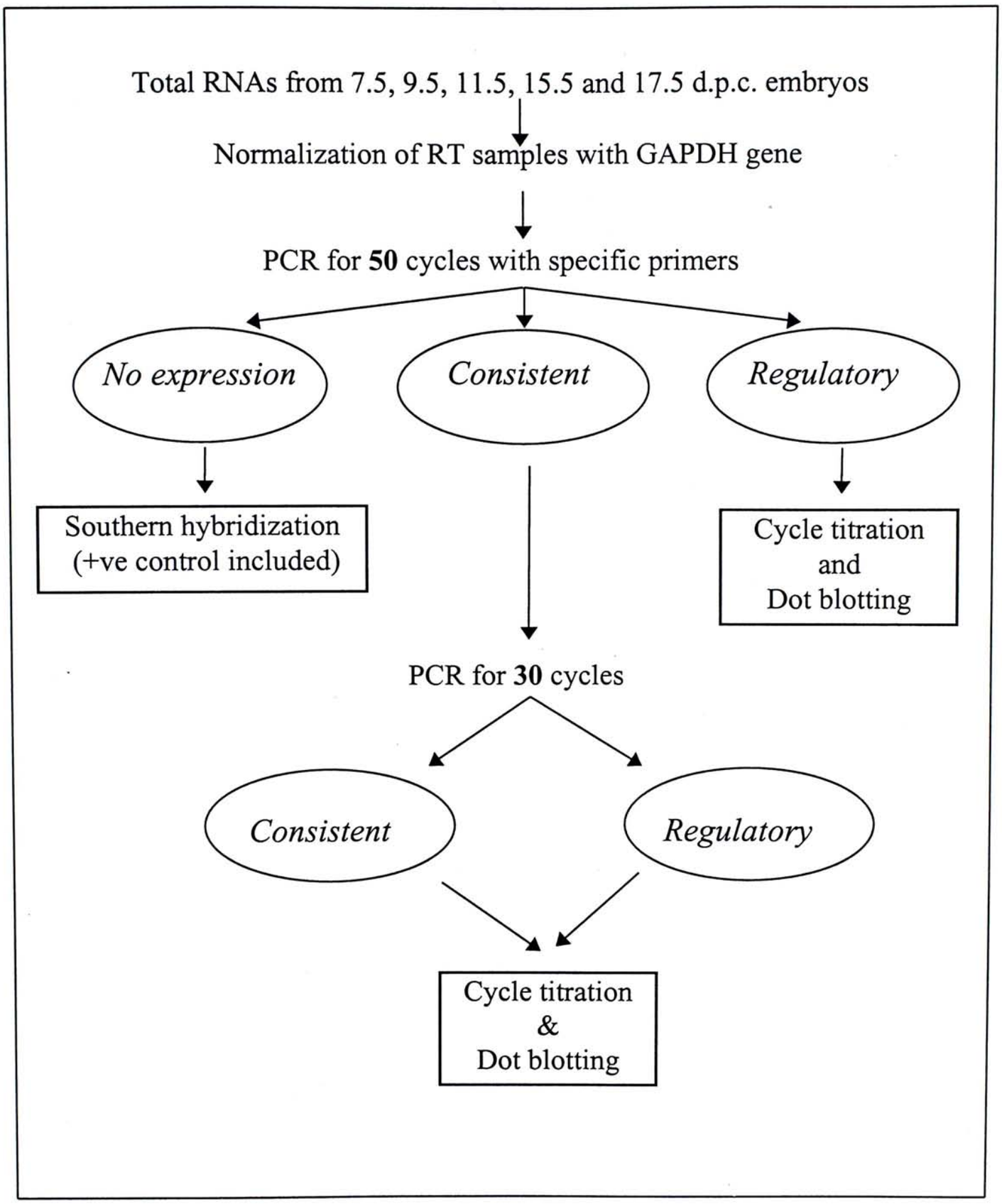


Figure 2.1 Schematic representation of the analysis procedures.

2.1.2 Design of primers

The design of primers for different gene sequences was facilitated by an automated primer search software OLIGO 3.4 and governed by the following criteria:

1) No inter- or intra-strand complementary base pairing, especially at 3' ends of the primers

2) No secondary priming site with sufficient homology, so that only one PCR product can be generated

3) Region of target sequence with internal secondary structure should be avoided

4) Length ranges from 20 - 25 nucleotides such that they have similar melting temperature

5) Primer specifying an amplified segment of 300 to 700 bp in size

6) Priming sites not further than 2 - 3 kb from the 3' end of the mRNA

7) Primer flanking target sequence containing an intron-exon border so that PCR product derived from genomic DNA will be greater in length

Fidelity of those amplified sequences of each cytokine or receptor gene by specific upper and lower primers was checked by hybridization to an oligonucleotide probe priming to the internal sequence flanked by the pair of primers. Sequence of the internal probes were designed in a similar fashion with PCR primers.

2.2 Materials

2.2.1 Chemicals and Reagents

1 Kb DNA marker, 1.0 μg/μl	GIBCO/BRL (No. 15615-016)
2-Mercaptoethanol	Sigma (No. 7154)
Agarose, type I: low EEO	Sigma (No. A-6013)
Blocking reagent	Boehringer Mannheim (No. 1096 176)
Bromophenol blue	Bio-Rad (No. 161-0404)
Cesium chloride (CsCl)	Sigma (No. C-4036)
Diethyl pyrocarbonate (DEPC)	Sigma (No. D-5758)
Dithiothretol (DDT)	GIBCO/BRL (No. 28025-013)
dNTPs	Pharmacia Inc. (No. 27-2035)
Absolute ethanol, analytical grade	Ajax chemicals (No. 214)
Ethidium bromide	Sigma (No. E-8751)
Ethylene diamine tetraacetic acid (EDTA)	Sigma (No. E-5134)
Ficoll	Sigma (No. F-4375)
Guanidinium thiocyanate	Fluka Biochemika (No. 50990)
Hydrochloric acid (HCl), 36%	Ajax chemicals (No. 1367)
Lithium chloride (LiCl)	Sigma (No. L-8895)
Magnesium chloride ($MgCl_2$), 25mM	Advanced Biotechnologies (No. AB-0288)
Maleic acid	Fluka Biochemika (No. 63180)
N-lauroylsarcosine, sodium salt	Sigma (No. L-5125)
Nylon membrane, positively charged	Boehringer Mannheim (No. 1029 299)
Oligo $d(T)_{12-18}$	Pharmacia Inc. (No. 7858)
Potassium chloride (KCl)	Sigma (No. 4504)
Potassium phosphate (KH_2PO_4)	Sigma (No. 5379)
Sodium acetate (NaOAc)	Sigma (No. S-8750)
Sodium chloride (NaCl)	Sigma (No. S-9625)
Sodium citrate, trisodium salt: dihydrate	Sigma (No. S-4641)
Sodium hydroxide (NaOH)	Sigma (No. S-5881)
Sodium dodecyl sulfate (SDS)	Sigma (No. L-5750)
Sodium phosphate (Na_2HPO_4)	Sigma (No. S-0876)
Synergel	Diversified Biotech Inc. (No. Syn 100)
Tris base	Sigma (No. T-8524)
Tween 20, EIA purity	Bio-Rad (No. 170-6531)
Xylene cyanol FF	Bio-Rad (No. 161-0423)

Remark: The water used throughout the experiments was of ultrapure grade.

2.2.2 Enzymes

Moloney murine leukemia virus reverse transcriptase (200U/μl) was obtained from GIBCO/BRL (No. 28025-013)

Recombinant RNasin ribonuclease inhibitor (40U/μl) was obtained from Promega (No. N2511)

Thermoprime^{plus} DNA polymerase (5U/μl) was obtained from Advanced Biotechnologies (No. AB-0301)

2.2.3 Buffers

5x First strand buffer

It was obtained from GIBCO/BRL which contained 250mM Tris-HCl (pH 8.3), 375 mM KCl and 15mM $MgCl_2$.

Buffer 1 (Maleic acid buffer)

Buffer 1 contained 0.1M maleic acid, 0.15M NaCl. The pH was adjusted to 7.5 with solid NaOH.

Buffer 2 (Blocking solution)

This buffer was prepared by diluting the blocking reagent stock (see page 47) 1:10 in buffer 1.

Buffer 3 (Detection buffer)

The buffer was composed of 0.1M Tris-HCl (pH 9.5), 0.1M NaCl and 50mM $MgCl_2$.

Washing buffer

The buffer was made from maleic acid buffer (buffer 1) added with 0.3% (v/v) Tween 20.

Gel-loading buffer (10x)

The buffer contained 0.42% bromophenol blue, 0.42% xylene cyanole FF and 25% Ficoll in sterilized water. It was stored at room temperature.

$TE_{0.1}$ buffer

$TE_{0.1}$ buffer contained 10mM Tris-HCl (pH 7.5) and 0.1mM EDTA (pH 8.0).

TAE buffer (Tris-acetate-EDTA buffer), Amresco

25x stock solution was diluted before use. 1x buffer contained 0.04M Tris base, 0.04M acetate and 0.001M EDTA.

Thermoprime^{plus} DNA polymerase reaction buffer IV (10x), Advanced Biotechnologies

The buffer contained 200mM $(NH_4)_2SO_4$, 750mM Tris-HCl (pH 9.0) and 0.1% (w/v) Tween

2.2.4 Solutions

Blocking reagent stock solution (10x conc.)

10 g of blocking reagent was dissolved in 100 ml buffer 1. The solution was heated to facilitate dissolution before autoclaving. The stock solution was stored at -20°C for long-term storage.

Ethidium bromide solution

1g of ethidium bromide was added to 100 ml of H_2O. The bottle was wrapped with aluminium foil and stored at room temperature.

Guanidinium thiocyanate solution

4M guanidinium thiocyanate and 0.1M Tris-HCl pH 7.5 in DEPC-treated water. The solution was stored at room temperature. 0.143 ml 2-mercaptoethanol (14M stock solution, Sigma) was added to 20 ml of the guanidinium thiocyanate solution immediately before use.

Hybridization solution

Hybridization solution was composed of 5x SSC, 1% (w/v) blocking reagent, 0.1% N-lauroylsarcosine and 0.02% SDS. It was stored at 4°C.

PBS (Phosphate-buffered saline)

8g of NaCl, 0.2g of KCl, 1.44g of Na_2HPO_4 and 0.24g of KH_2PO_4 were dissolved in 800 ml of distilled H_2O and the pH was adjusted to 7.4 with HCl. Water was added to make 1 litre buffer which was then sterilized by autoclaving.

SDS (10%)

100g of SDS was dissolved in 1000 ml of H_2O which was heated to assist dissolution. The pH was adjusted to 7.2 with a few drops of concentrated HCl.

SSC (20x)

0.3M NaCl and 0.03M sodium citrate. 175.3g of NaCl and 88.2g of sodium citrate were dissolved in 800 ml of H_2O. The pH was then adjusted to 7.0 with a few drops of a 10N solution of NaOH. Water was added to make 1 liter solution which was sterilized by autoclaving.

Tris-HCl, 1M (pH 7.5)

121g of Tris base was dissolved in 800 ml H_2O and pH was adjusted to 7.5 with concentrated HCl. Water was added to make 1 liter.

2.2.5 Probe labeling and detection kits

DIG Oligonucleotide 3'-end labeling kit	Boehringer Mannheim (No. 1362372)
DIG luminescent detection kit	Boehringer Mannheim (No. 1363514)

2.2.6 Primers and internal probes

Table 2.1 Sequence of primer pairs and internal probes specific for mouse cytokines.

Cytokine		Sequence (5' to 3')	size (bp)
GAPDH	upper primer	ACCACAGTCCATGCCATCAC	452
	lower primer	TCCACCACCCTGTTGCTGTA	
IL-1α	upper primer	ACAGTATCAGCAACGTCAAGCAA	
	lower primer	CCGACTTTGTTCTTTGGTGGCA	546
	internal probe	GGCAACTCCTTCAGCAACACG	
IL-1β	upper primer	GAGCTTCAGGCAGGCAGTATC	
	lower primer	GTATAGATTCTTTCCTTTGAGGC	382
	internal probe	CACTTGTTGGTTGATATTCTGTC	
IL-1R tI	upper primer	ATGGAAGGGATGACTATGTTGGA	
	lower primer	GCTGCAGCCTCTTATGATGGG	506
	internal probe	TGATCTCCGTTGGGCTGGCA	
IL-1R tII	upper primer	AAGGAACAACCACGGAACCCAT	
	lower primer	AGCCCTGCGTTTACACCGTCT	446
	internal probe	CGCAATGCTCCAGGAGAACGT	
IL-2	upper primer	AGCAGCTGTTGATGGACCTAC	
	lower primer	GCAGGAGGTACATAGTTATTGAG	380
	internal probe	TCCTCAGAAAGTCCACCACAGT	
IL-2Rα	upper primer	ATTCCGGGATACAAGGCTCTAC	
	lower primer	TGTTGCCAGGTGAGCCCGCT	332
	internal probe	GAAGTGTGGGAAAACGGGGTG	
IL-3	upper primer	ATAGGGAAGCTCCCAGAACCTGAACTC	
	lower primer	AGACCCCTGGCAGCGCAGAGTCATTC	207
	internal probe	ATTCGCAGATGTAGGCAGGCA	
IL-3R (AIC2A)	upper primer	TACACACGATTTTCTAATGGAGATA	
	lower primer	TAGATGCTGTTGGGTAGGAATAG	326
	internal probe	GAGCTGAAGGAGGAACCTGAC	
IL-3R (AIC2B)	upper primer	ATACACGATTTTCCATCACAAACG	
	lower primer	TAGATGCTGTTGGGTAGGAATAG	325
	internal probe	GAGCTGAAGGAGGAACCTGAC	
IL-4	upper primer	TGACGCACAGAGCTATTGATGG	
	lower primer	ATGATGCTCTTTAGGCTTTCCAG	422
	internal probe	AGCTGGGGGTTGAGACC	

Table 2.1 Sequence of primer pairs and internal probes specific for mouse cytokines. **(Cont'd)**

Cytokine		Sequence (5' to 3')	size (bp)
IL-5	upper primer	TTGACAAGCAATGAGACGATGAG	
	lower primer	CAGTTTGAGGCCAGCCTGCG	507
	internal probe	TCCCTACTCATAAAAATCACCAG	
IL-6	upper primer	TGAGAAAAGAGTTGTGCAATGGC	
	lower primer	GAATGTCCACAAACTGATATGCTT	479
	internal probe	GTTAGGAGAGCATTGGAAATTGG	
IL-10	upper primer	TAGAGCTGCGGACTGCCTTCA	
	lower primer	TCATGGCCTTGTAGACACCTTG	351
	internal probe	GGAGAAATCGATGACAGCGCC	
IL-11	upper primer	CTAGCTGCACAATGAGAGAC	
	lower primer	CCAGTCCAAGGTCAGGTGCA	396
	internal probe	GATGCTTCCCCAGGCTGAGG	
LIF	upper primer	TCTCTTCATTTCCTATTACACAGC	
	lower primer	GACCACCACACTTATGACTTGC	415
	internal probe	CACGGTACTTGTTGCACAGAC	
LIFR-l	upper primer	TCATCACCACCTTCGAAAATAGC	
	lower primer	CCGCATATTTTAAGTGACCGCT	415
	internal probe	TGGTGCAACTCATCTCGGTCT	
LIFR-s	upper primer	TCATCACCACCTTCGAAAATAGC	
	lower primer	GCGCAACAATGGGAGCCAGT	340
	internal probe	TGGTGCAACTCATCTCGGTCT	
gp130	upper primer	ATATTGCCCAGTGGTCACCTCA	
	lower primer	CACCGTCTACACTATCCACCAG	429
	internal probe	CGGACCTTGAGAACACTTGCA	
SCF-l	upper primer	TGGACAAGTTCTCAAATATTTCT	313
	lower primer	GCAACAGGGGGTAACATAAAT	
SCF-s	upper primer	TGGACAAGTTCTCAAATATTTCT	281
	lower primer	CTTTGCGGCTTTCCCTTTCT	
	* *internal probe*	CGAAGAGGCCAGAAACTAGATC	
c-kit	upper primer	GCGGATCACAAAGATTTGCGATT	
	lower primer	CGAGTTGACCCTCACGGAATG	472
	internal probe	CCCAGGGATGCCGGTCGAC	

**internal probe* specific to both forms of SCF.

Table 2.1 Sequence of primer pairs and internal probes specific for mouse cytokines. **(Cont'd)**

Cytokine		Sequence (5' to 3')	size (bp)
M-CSF	upper primer	GTAGCCACATGATTGGGAATGG	
	lower primer	TCATGGAAAGTTCGGACACAGG	304
	internal probe	GTTCTGCTCCTCATAGTCCTTG	
c-fms	upper primer	TGCTCCACTTCTCCAGCCAAGTGGCTC	
	lower primer	AGGTGTTCACTGGAGCTCTCCTCTTCTG	607
	internal probe	TGGAGGAGGAAGCAGATCTGT	
GM-CSF	upper primer	TGGTCTACAGCCTCTCAGCAC	
	lower primer	AAGGGGATATCAGTCAGAAAGGT	364
	internal probe	TAAGGCTGTCTATGAAATCCGCA	
G-CSF	upper primer	ATCCAGGCCAGCGGCTCGG	
	lower primer	GGGCTTTCTGCTCAGGTCTAG	467
	internal probe	AGAAGTGAAGGCTGGCATGGC	
CNTF	upper primer	CTGGCTAGCAAGGAAGATTCGT	
	lower primer	ATGAGAAGAAATGACACGGAGGT	484
	internal probe	TCTCCAATTGTGACAGGCATCC	
IFN-γ	upper primer	AGGAACTGGCAAAAGGATGGTG	
	lower primer	GTGCTGGCAGAATTATTCTTATTG	353
	internal probe	TCCTGCAGAGCCAGATTATCTC	
TGF-β	upper primer	CATGGAGCTGGTGAAACGGAAGCGCAT	
	lower primer	CTCCAGTGACGTCAAAAGACAGCCACT	497
	internal probe	GTCCAAACTAAGGCTCGCCAG	
TNF-α	upper primer	TCCCCAAAGGGATGAGAAGTTC	
	lower primer	TCATACCAGGGTTTGAGCTCAG	411
	internal probe	CACACTCAGATCATCTTCTC	
TNF-β	upper primer	AGCCCATCCACTCCCTCAGAA	
	lower primer	ATGCCGTCGGTGTGGGTGGA	423
	internal probe	TTGTTGCTCAAAGAGAAGCCATG	

2.3 Methods

2.3.1 Preparation of total RNA from mouse embryos at different stages

a. Mice dissection for embryo

Pregnant ICR mice were obtained from the Animal House of the Chinese University of Hong Kong. Embryos were isolated from pregnant mice of 7.5, 9.5, 11.5, 15.5 and 17.5-day postcoitum (d.p.c.). Deciduum (from 7.5 d.p.c. embryos) and placenta (from 9.5, 11.5, 15.5 and 17.5 d.p.c. embryos) were isolated separately.

b. Guanidinium thiocyanate cell lysate

Each tissue was first washed in cold PBS and was passed through a 0.1 cm sterile stainless steel mesh. The cell suspension was then centrifuged and the resulted cell pellet was resuspended in 0.5 ml PBS. The suspension was immediately added dropwise to appropriate volume of guanidinium thiocyanate (GT) solution with continuous vortexing. Vigorous mixing for rapid and complete homogenization was necessary for efficient protein denaturation with consequent elimination of nucleolytic damage to the RNA molecules. (Herrmann and Frischauf, 1987). The amount of GT solution was based on an estimation of 1.0×10^9 cells in 40 ml GT solution. The cell lysate was stored at -70°C until it was further processed.

c. Isolation of RNA by centrifugation through CsCl gradient

RNA was isolated using a modified guanidinium thiocyanate/cesium chloride method in which RNA was separated from the GT homogenate by ultracentrifugation through a dense cushion of CsCl (Chirgwin *et al*, 1979). The frozen cell lysate was first thawed by incubating in a 65°C waterbath with vigorous shaking. After complete thaw, it was further heated at 65°C for 5 min and then quickly chilled on ice. By passing through a 25-gauze needle for several times, large DNA molecules were sheared. This step prevented the formation of impenetrable DNA mat which would otherwise block the sedimentation of RNA molecules and thereby facilitating the purification of RNA (Herrmann and Frischauf, 1987). 2 ml of each sample was then layered on top of a 1 ml cushion of 5.7M CsCl in a DEPC-treated thin-walled polyallomer tube (Beckman, No. 328874) and centrifuged in a SW 60 Ti rotor at 32,000 rpm for 18 hours at 18°C.

After centrifugation, supernatant was removed by aspiration while the cushion was discarded by inverting the tube very quickly. The RNA pellet which should be translucent and located at the bottom of the tube was allowed to dry thoroughly in the inverted tube. The bottom 0.5 cm of the ultracentrifuge tube was then cut off with a new sterile scalpel blade and the RNA pellet was subsequently rinsed out and resuspended in total 400 µl DEPC-treated water and transferred to a new microfuge tube. Complete dissolution of RNA was achieved by vigorous vortexing for a few minutes. The RNA suspension was transferred to another new microfuge tube after brief spinning. RNA was then precipitated with 0.3M NaOAc and 2.5 volumes of

absolute ethanol. The tube was centrifuged at 13,000 g for 30 min at room temperature. The RNA pellet was washed with 70% and absolute ethanol sequentially and dried under vacuum.

d. Spectrophotometric determination of RNA amount

The amount of RNA was estimated by spectrophotometric measurement at OD_{260}. Absorbance of RNA was measured by GeneQuant, RNA/DNA calculator (Pharmacia, No. 80-2103-93). One OD unit corresponds to approximately 40 μg/ml for single-stranded RNA and so concentration of RNA equals to 40 μg/ml x dilution factor x OD_{260}. The ratio between the readings at 260nm and 280nm provides an estimate of the purity of the RNA. Pure preparations of RNA should have OD_{260} / OD_{280} values of 2.0 (Maniatis *et al.*, 1982).

2.3.2 Preparation of embryo sections

Several freshly isolated embryos from each stage were fixed in cold Bouin's fluid for 24 hours. The cold Bouin's fluid would avoid the rapid autolysis of the organ. Dehydration was carried out in an ascending concentration series of ethanol (70%, 80%, 95% and 2 changes for 100%). The embryos were further cleared in xylene and later embedded in Paraplast paraffin wax (m.p. 56-58°C). The embedded specimens were sectioned with microtome (Leica 2035 Biocut) into sections of 5 μm thickness which were fixed on glass slide with albumin. The sections were stained with Mayer's hematoxylin and eosin (H & E staining).

2.3.3 Primers and internal probes

Oligonucleotide primers and internal probes were custom synthesized by Integrated DNA Technologies, Inc. The oligonucleotides were purified by gel filtration column and obtained as lyophilized powder.

Upon arrival, powder of primer was first dissolved in 500 µl $TE_{0.1}$ buffer and the concentration was determined by OD measurement at 260 nm according to the following formula:

Concentration of oligonucleotide (pmol/ µl) = OD_{260} / ε x dilution factor x 1000

where ε corresponds to the summation of extinction coefficient of the bases in an oligomer.

(A = 11.5, T = 8.8, G = 11.7, C = 7.3)

A working solution of 25 pmol/ µl of each primer was prepared.

2.3.4 Cytokine mRNA Phenotyping by Reverse transcription-Polymerase chain reaction

a. Reverse transcription (First strand cDNA synthesis)

RNA was denatured at 65°C for 5 min and then chilled on ice. It was subsequently reverse transcribed into cDNA in a 20-µl standard reaction mixture containing 1µg of total cellular RNA, 1x Gibco/BRL First strand buffer (50mM Tris-HCl, pH 8.3, 75mM KCl and 3mM $MgCl_2$), 40 U of RNasin (Promega), 0.1 µg Oligo $d(T)_{12-18}$, 10mM DTT, 0.2mM of each dNTP and 200 U of Moloney murine leukemia virus reverse transcriptase (Gibco/BRL). The reaction mixture was incubated at 37°C for 60 min and the reaction was terminated by immediate chilling on ice and was stored at -20°C until next procedure.

b. Polymerase chain reaction (PCR)

cDNAs of interest were amplified by PCR using specific primers. The reverse-transcribed (RT) products were first denatured by boiling for 10 min and then quickly chilled on ice just before PCR. The amplification was done in a total volume of 50 µl with denatured RT sample equivalent to 0.1 µg of total RNA, 1x PCR buffer [20mM $(NH_4)_2SO_4$, 75mM Tris-HCl, pH 9.0 and 0.01% (w/v) Tween], 25 mM $MgCl_2$, 10mM of each dNTP, 1 µM of each upper and lower primers and 0.25 U of Thermoprime^{plus} DNA polymerase (Advanced Biotechnologies). The mixture was overlaid with mineral oil (Sigma) and then amplified with the PTC-100 Programmable Thermal controller

(MJ Research, Inc.), using profile of denaturation at 94°C for 1 min, primer annealing at 5-10°C below its melting temperature for 1 min and extension at 72°C for 1 min. The samples were stored at -20°C before analysis.

2.3.5 Analysis of PCR products with agarose gel electrophoresis

PCR products were separated and analyzed in agarose gel by electrophoresis. DNA molecules ranged from 100 to less than 1000 bp were electrophoresed in a gel mixture of 0.7% (w/v) agarose and 1.5% Synergel (a novel gelling and sieving agent for improving agarose gel performance). The Synergel-agarose binary gel was the functional equivalent of 3.5% (w/v) agarose.

Buffer used for gel preparation and electrophoresis was 1x TAE buffer containing 0.5 μg/ml ethidium bromide. Usually, 10 or 15 μl of each sample was subjected to electrophoresis at 5-10 V/cm of gel. Migration of the tracking dyes was an indicator of the separation process which was stopped when the first dye front (bromophenol blue) reached two-third of the gel length. EtBr-stained DNA molecules were visualized with a UV transilluminator. The EtBr-nucleic acid complexes emit orange red fluorescent light upon UV irradiation. The gel was then photographed with Polaroid (MP-4) land camera with Polaroid 667 films (ASA 3000).

2.3.6 Analysis of PCR products with Southern blotting

Cytokine gene products after 50-cycle PCR which could not be visualized in agarose gel were considered absent in the initial template. In other words, those cytokines did not express in corresponding embryo tissues. The result was verified by Southern blotting using oligonucleotide probes corresponding to the internal sequences of the target. A positive control for each 'no expression' cytokine was included.

a. DNA transfer from gel to nylon membrane

Samples, together with a positive control, were electrophoresed in 2% agarose gel and stained with EtBr as described in previous section (*Section 2.3.5*). The gel was then photographed with a fluorescent scale under UV light. Unused areas of the gel were cut off with a clean scalpel blade on a piece of Saran wrap. The DNA in the gel was subsequently depurinated in 2 gel volumes of 0.2M HCl for 10 min at room temperature with gentle shaking and rinsed briefly with double distilled water. The DNA was then ready for transfer to a positively charged nylon membrane by capillary blotting at alkaline pH.

The blotting apparatus was assembled as shown in **Figure 2.2**. The transfer solution was 0.4M NaOH. Any air bubbles between gel and membrane or 3MM paper above were removed by rolling a clean pipette over the surfaces. The edge of gel was surrounded with Saran wrap to prevent 'short circuits', in which transfer solution flows directly from the reservoir to the dry paper towels above the membrane, rather than

passing through the gel. The transfer was done for 4 hours and the set-up was disassembled. Remains of the gel was restained with EtBr (1 μg/ml) to see if any DNA remained in it. The membrane was rinsed in 2x SSC solution and then air-dried at room temperature. The dried membrane was finally placed between two 3MM paper sandwiched within two glass plates and baked at 120°C for 30 min to fix the DNA on it. After fixation, the membrane was stored at room temperature in dry environment prior to hybridization.

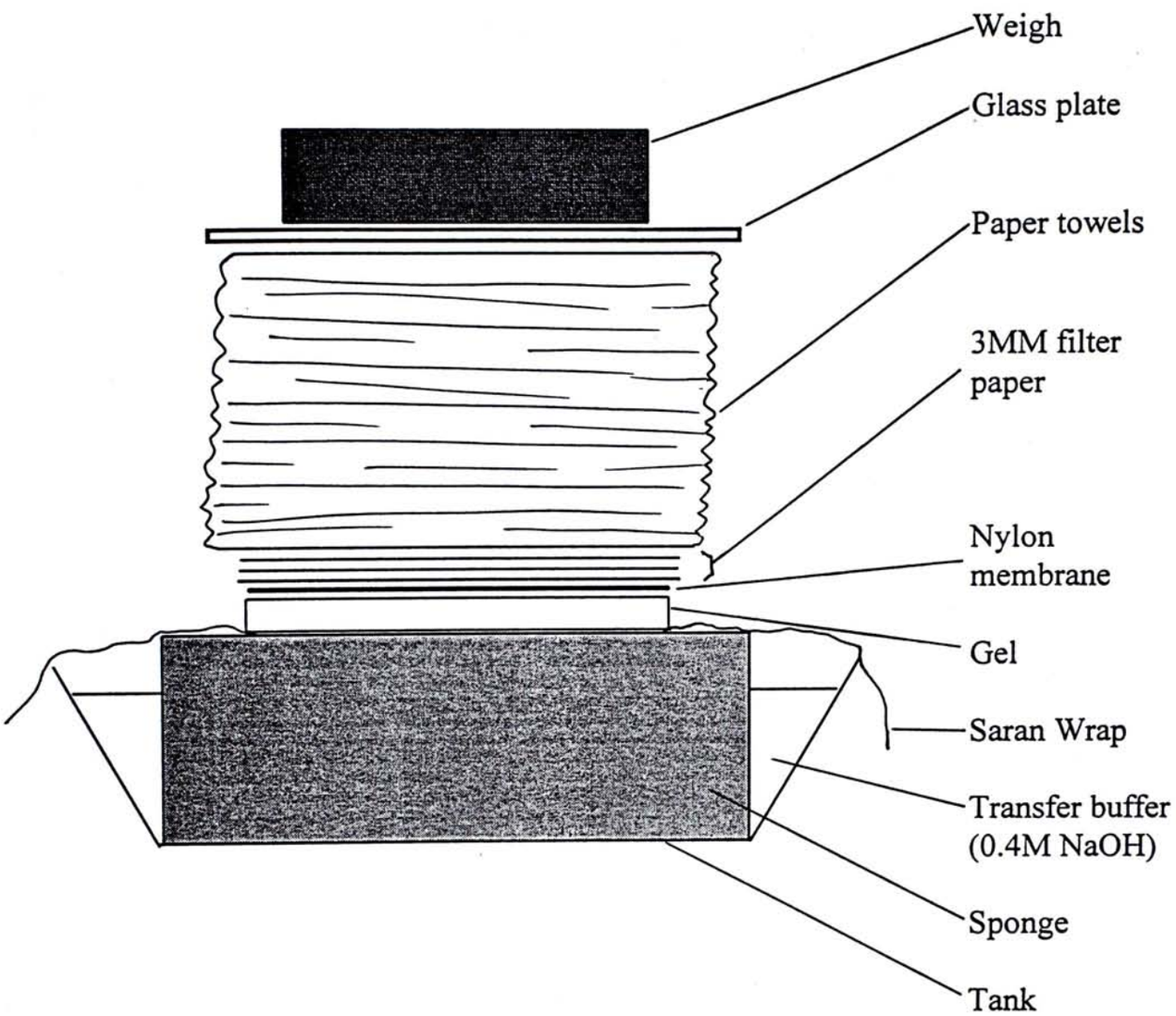


Figure 2.2 Assembly of Southern blotting.

b. Probe labeling

Oligonucleotides probes were labeled at their 3' end with terminal transferase by the incorporation of a single digoxygenin-labelled dideoxyuridine-triphosphate (DIG-ddUTP). The labeling reaction was performed according to the instruction manual (Boehringer Mannheim). In brief, 4 μl of 5x reaction buffer, 4 μl of 25mM $CoCl_2$ solution, 100 pmol oligonucleotides, 1 μl of 1mM DIG-ddUTP solution, 1 μl of 50 U/ μl terminal transferase were mixed in a microcentrifuge placed on ice. Sterile water was added to make final volume of 20 μl. The mixture was then incubated at 37°C for 15 min and chilled on ice afterwards. 1 μl of 20 mg/ml glycogen solution was mixed with 200 μl 0.2mM EDTA solution (pH 8.0) and then 2 μl of the dilution was added to the tube to stop the reaction. The labeled oligonucleotide was precipitated with 2.5 μl 4M LiCl and 75 μl prechilled (-20°C) ethanol for 30 min at -70°C, recovered by centrifugation at 13,000g for 15 min. The pellet was washed with 50 μl cold 70% ethanol, vacuum dried and finally resuspended in 50 μl sterile water. The labeled probe was stored at -20°C before use.

c. Prehybridization

The baked membrane was placed into a roller bottle, which was later filled with at least 20 ml hybridization solution per 100 cm^2 of membrane for 2 hours at the hybridization temperature with constant rotation in a hybridization incubator (Robbins Scientific Co., Model 1000).

The hybridization temperature depended on the length and sequence of the oligonucleotide probes. Hybridization was carried out at 5-10°C below the melting temperature (T_m) of the probe. Assuming 2°C for A-T bond and 4°C for G-C bond, T_m of a synthetic oligonucleotides was estimated by summing up the total number.

[i.e. $T_m = (A-T)x2\,°C + (G-C)x4°C$]

d. Hybridization

After the prehybridization process, the solution was replaced by about 2.5 ml per 100 cm^2 membrane of hybridization solution. 3' end-DIG labeled oligonucleotide probe was subsequently added to the hybridization fluid to a final concentration of 10 pmol/ml. Hybridization was carried out at a particular hybridization temperature for 16 hours with constant rotation. The membrane was then washed twice with 0.5 x SSC/ 0.1% (w/v) SDS for 15 min. The washed membrane was directly used for detection of hybridized oligonucleotides.

e. Detection of DIG-labeled probe

The hybridized probe was detected using DIG luminescent detection kit from Boehringer Mannheim. The procedures followed the instructions of the kit. Briefly, the DIG-labeled probe were first immunodetected with anti-DIG, Fab fragments conjugated to alkaline phosphatase and were then visualized with the chemiluminescence substrate CSPD. Enzymatic dephosphorylation of CSPD by alkaline phosphatase led to a light emission at a maximum wavelength of 477 nm

which was recorded on X ray films. The intensity of the hybridization signals was evaluated by the imaging densitometer (Bio-Rad Model GS-670).

2.3.7 Cycle titration of PCR and dot blotting of regulatory cytokine mRNA

a. Cycle titration of PCR

Cytokines which showed regulation at different stages of embryonic development as observed in agarose gel electrophoresis after 30-cycle PCR were studied with semi-quantitative PCR. It was achieved by titrating PCR against different cycles of amplification which were within the exponential phase of PCR. The reactions were prepared as a master mixture to enhance accuracy. The conditions of reaction were the same as described in *Section 2.3.4 b* and subjected to 5, 10, 15, 20, 25 and 30 cycles of amplification. The PCR products were kept at -20°C prior to analysis by dot blotting.

b. Dot blotting

The PCR products were verified and quantified by dot blotting on a nylon membrane and subsequent hybridization with specific internal probes. 5 - 15 μl of PCR products (varied with different sets of sample) was added to 200 μl of denaturing solution (0.2M NaOH) in an eppendorf tube and incubated for 15 minutes at room temperature. During the incubation period, the nylon membrane was fixed and pre-wetted with sterile water in a Bio-dot SF Microfiltration apparatus (Bio-Rad, No. 170-

6542) applied with gentle vacuum. Denatured samples were then loaded into wells and blotted on membrane with gentle suction force. Each sample was chased by adding 400 µl 20x SSC to each well and sucking it through. The nylon membrane was then dissembled from the apparatus and allowed to dry in air. DNA was fixed to membrane by baking at 120°C for 30 min.

The hybridization and detection procedures following dot blotting were the same as used with Southern blots (*Section 2.3.6 b-e*). The hybridization signal in dots were scanned and photographed by imaging densitometer (Bio-Rad Model GS-670) and video copy processor (Mitsubishi P68E) respectively.

2.4 Results

2.4.1 Sagittal sections of mouse embryos

The gestation period for the ICR mouse embryo is generally 19-20 days. Referring to **Figure 2.3**, embryo is in its early organogenesis on day 7.5 and 9.5 while it enters organogenesis on day 11.5. From 14 days onwards, a complete body plan of the embryo establishes and the fetus develops and grows in size (Hogan *et al.*, 1994). In this study, RNAs were extracted from embryos of mid- (7.5 days) to late- (17.5 days) gestation.

The rate at which embryo develops is hardly identical. Developmental differences between embryos of the same nominal age inevitably exist. The rate varies to certain degree from hatch to hatch or even from individual to individual within the same mother. Due to the minor differences, embryos from at least 3 mothers were pooled together and total RNAs were extracted from the pooled tissue. This practice minimized the individual difference.

Typical embryos from each selected stage (7.5, 9.5, 11.5, 15.5 and 17.5 d.p.c.) were preserved and sectioned laterally. The embryo sections were stained with Mayer's hematoxylin and eosin and were presented in **Figure 2.4**.

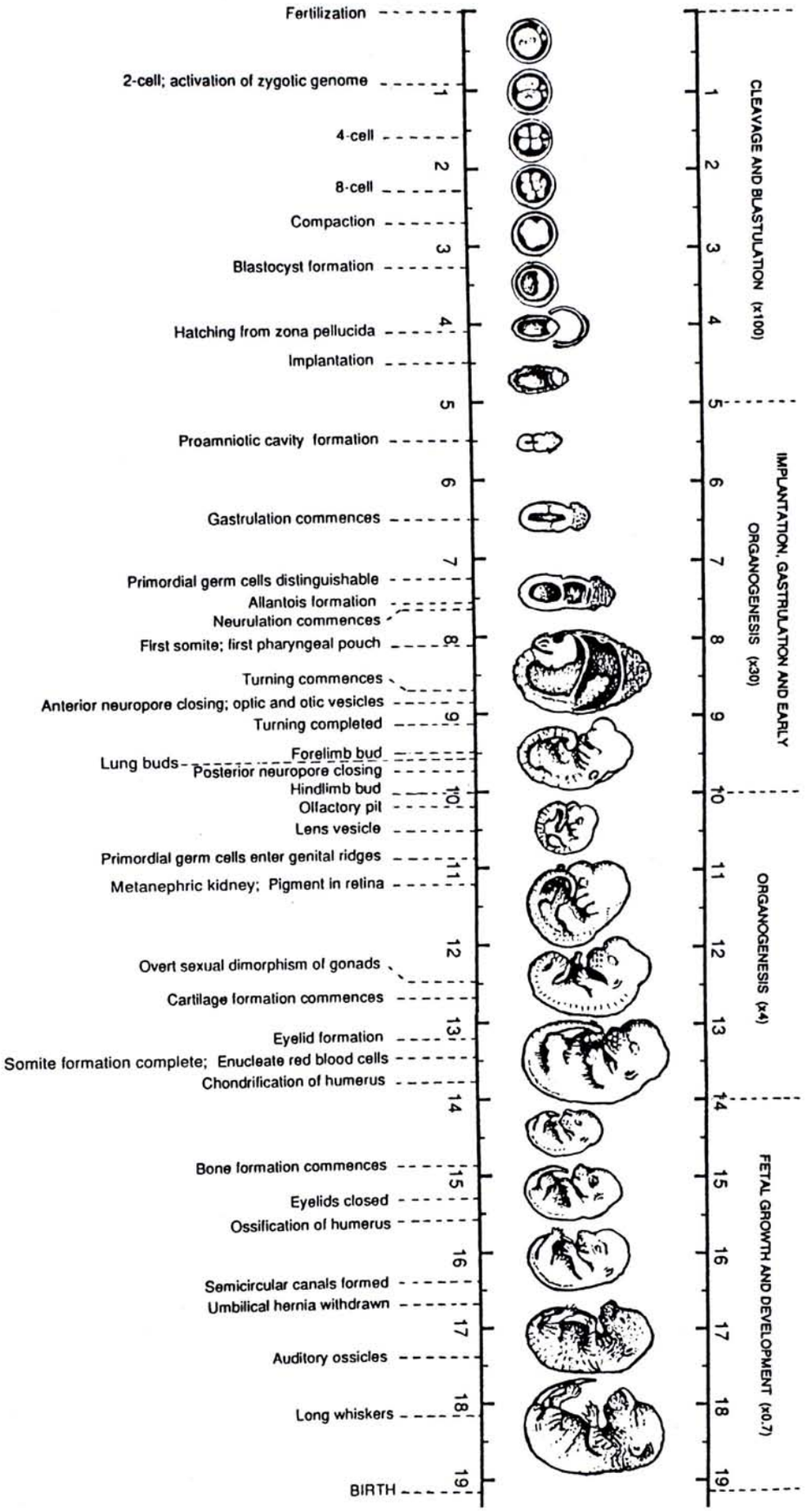


Figure 2.3 Time course of mouse embryonic development (adapted from Hogan *et al.*, 1994).

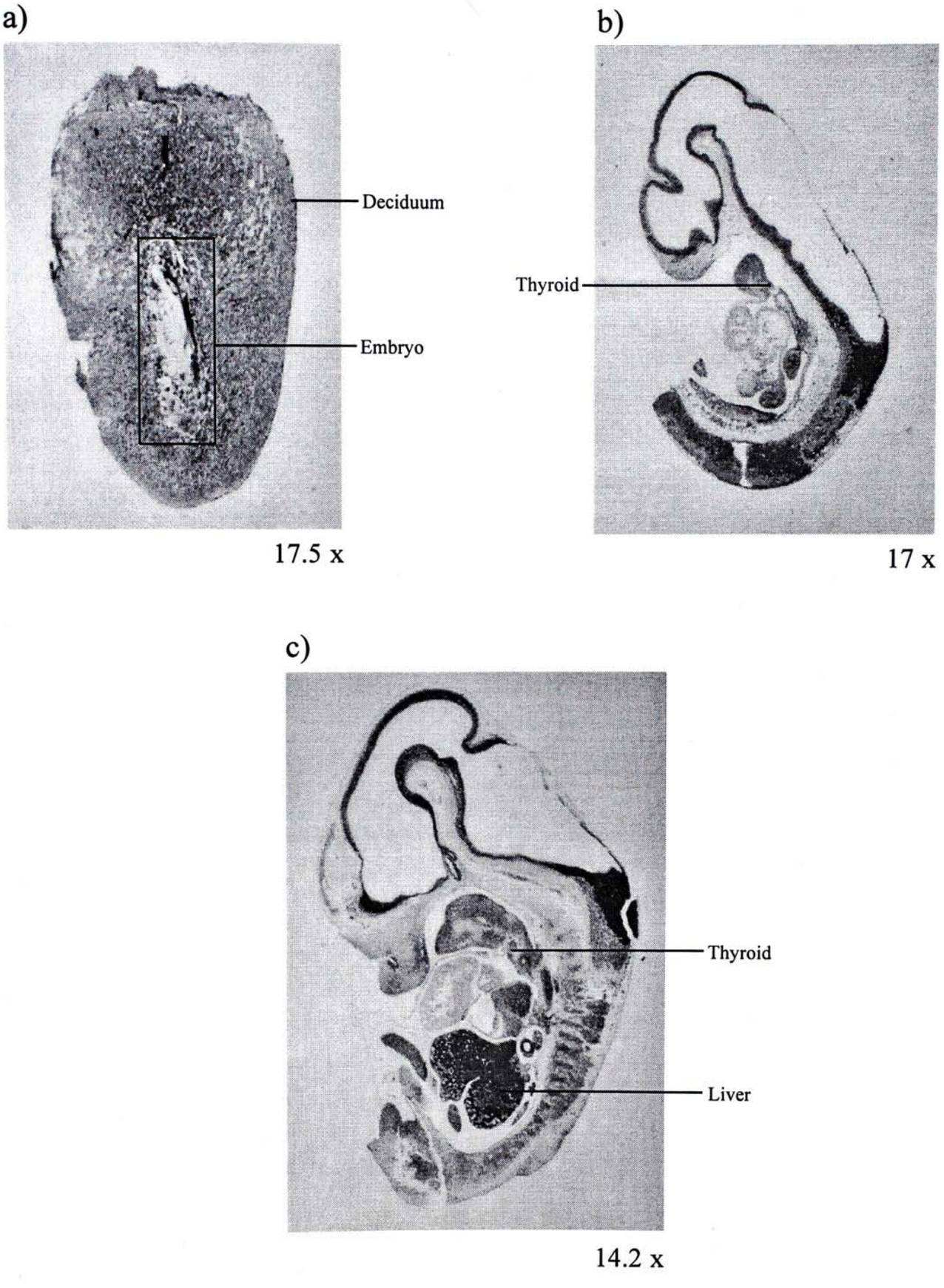


Figure 2.4 Sagittal sections of **mouse** embryo stained with hematoxylin and eosin.

a: 7.5 d.p.c. embryo in deciduum; **b**: 9.5 d.p.c. and **c**: 11.5 d.p.c. embryos.

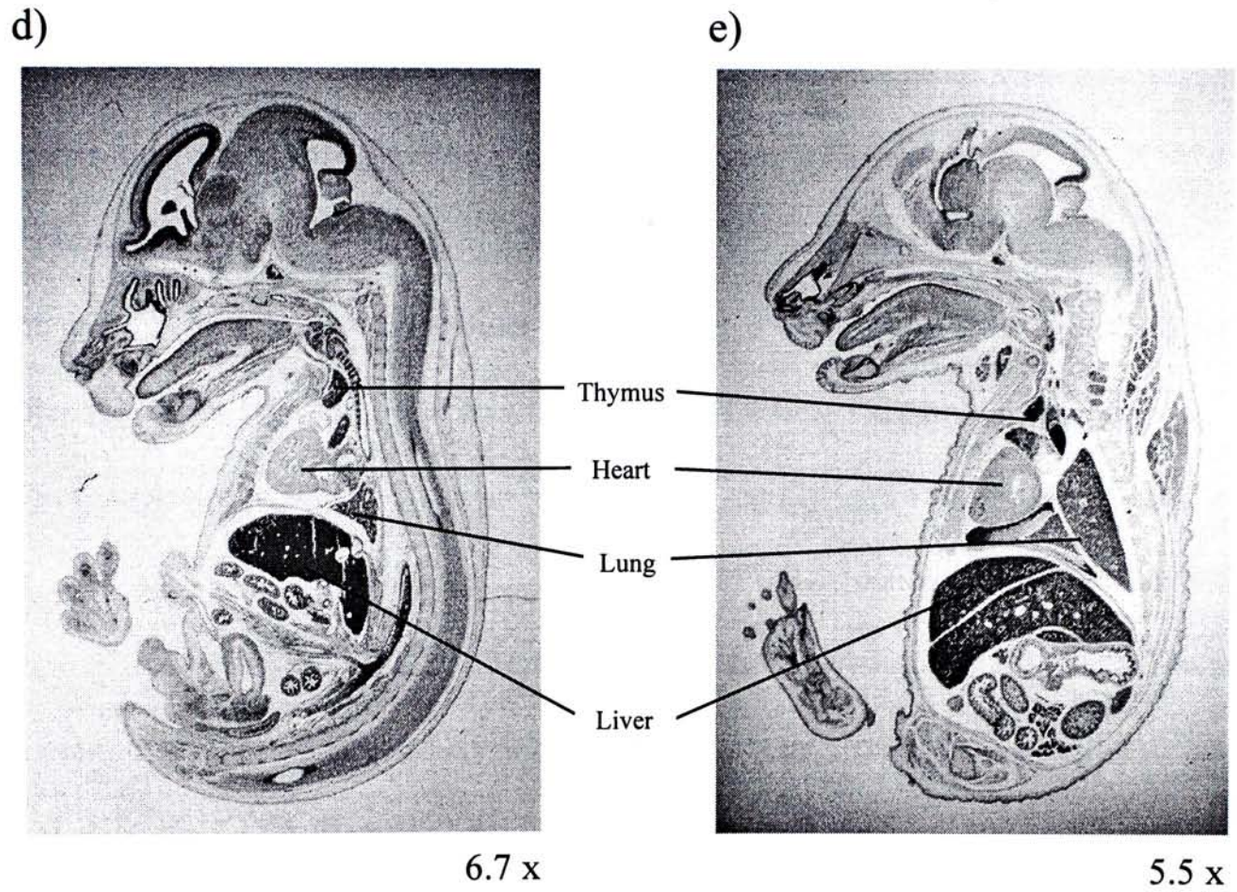


Figure 2.4 (cont'd) Sagittal sections of **mouse** embryo stained with hematoxylin and eosin.

d: 15.5 d.p.c. and **e**: 17.5 d.p.c. embryos.

On 7.5 d.p.c., amnion had been formed and allantois was appearing. The whole embryo was very small in size (2mm in length) and was encased in deciduum (**Figure 2.4a**). For embryo at this early stage, deciduum was taken as the mother tissue control. By 9.5days (**Figure 2.4b**), turning of embryo had taken place and heart began to beat (Hogan *et al.*, 1994). The length varied from 1.2 to 2.5mm. From this day onwards, condensation of the forelimb and hindlimb bud began. The extremities and tail were enlarging rapidly. By 11.5 d.p.c.(**Figure 2.4c**), a footplate had formed in the anterior limb bud. The posterior limb buds were not yet divided into leg and foot. These projecting appendages curved forward or inward (Theiler, 1972). Most embryos were 6-7mm long at this stage. The liver was growing rapidly. As seen from **Figure 2.4d**, the liver was well developed in 15.5 d.p.c. embryo and contained scattered blood-forming foci (Theiler, 1972). The length of embryos varied from 11.5 to 14mm. Separation of toes and fingers was prominent. They were clearly divergent and became parallel on day 17.5 (**Figure 2.4e**). The skin was wrinkled and thickened, and the subcutaneous veins were no longer distinctly visible. The length of this late stage embryo ranged from 16.5 to 20mm depending on the degrees of curvature of the fetuses.

2.4.2 Preparation of total RNA

RNA of mouse embryos and their placentas aged at 7.5, 9.5, 11.5, 15.5 and 17.5 d.p.c. were prepared by ultracentrifugation through CsCl gradient. The quantity of RNA was estimated by spectrophotometric measurement at OD_{260} and the results were

tabulated in **Table 2.2**. As embryo is in close connection with mother body, its cytokine expression level is easily contaminated with mother's contribution. Due to this reason, placenta at each time point was then taken as mother tissue control and its cytokine expression profile was also determined for comparison.

Table 2.2 Extraction of RNA from mouse **embryo** and **placenta** aged at 7.5, 9.5, 11.5, 15.5 and 17.5 d.p.c.. Two batches of each sample were prepared and labeled as B1 and B2.

A) Embryo RNA

	7.5		**9.5**		**11.5**		**15.5**		**17.5**	
	B1	B2	B1	B2	B1	B2	B1	B2	B1	B2
OD_{260}	0.024	0.409	0.948	1.357	0.333	1.848	0.226	1.901	0.195	0.481
OD_{280}	0.013	0.268	0.532	0.781	0.157	1.051	0.107	1.088	0.086	0.282
OD_{260}/OD_{280}	1.846	1.527	1.782	1.736	2.121	1.753	2.112	1.748	2.270	1.705
conc. (μg/μl)	0.19	1.64	3.79	5.43	2.66	7.37	1.81	7.60	1.56	1.92

B) Placenta RNA

	7.5		**9.5**		**11.5**		**15.5**		**17.5**	
	B1	B2	B1	B2	B1	B2	B1	B2	B1	B2
OD_{260}	0.687	0.502	0.369	0.264	0.138	0.422	0.648	0.708	0.482	0.400
OD_{280}	0.366	0.291	0.203	0.150	0.080	0.240	0.368	0.436	0.270	0.224
OD_{260}/OD_{280}	1.877	1.728	1.818	1.761	1.725	1.758	1.761	1.624	1.785	1.786
conc. (μg/μl)	2.75	2.01	1.48	1.06	0.55	1.69	2.59	2.83	1.93	1.60

2.4.3 Cytokine mRNA phenotyping

The expression profile of various cytokines was determined for 7.5, 9.5, 11.5, 15.5 and 17.5 d.p.c. mouse embryo and their respective placenta. For significant comparisons of cytokine mRNAs present in embryos, cDNA samples were first normalized by a constitutively expressed glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene. This normalization step allowed the use of equivalent amounts of cDNA for each assay. As shown in **Figure 2.5**, band intensities of PCR product (GAPDH) of all time-point samples were roughly the same. Cytokine mRNA phenotyping was carried out only after RT samples were equalized.

According to the analysis procedures depicted in **Figure 2.1**, phenotyping started with 50-cycle-PCR using 30 pairs of specially designed primers for different cytokine or receptor genes. Intensity of the resulted DNA bands as visualized in agarose gel provided a means of comparison of mRNA quantity among the 5 developmental stages. To achieve semi-quantitative assessment, cycle number of PCR should lie on the exponential phase of amplification. Therefore, cycle number for those cytokines which gave consistent band intensity was further lowered down to thirty. The results for the 30 cytokine and cytokine receptor genes were summarized in **Figure 2.6**.

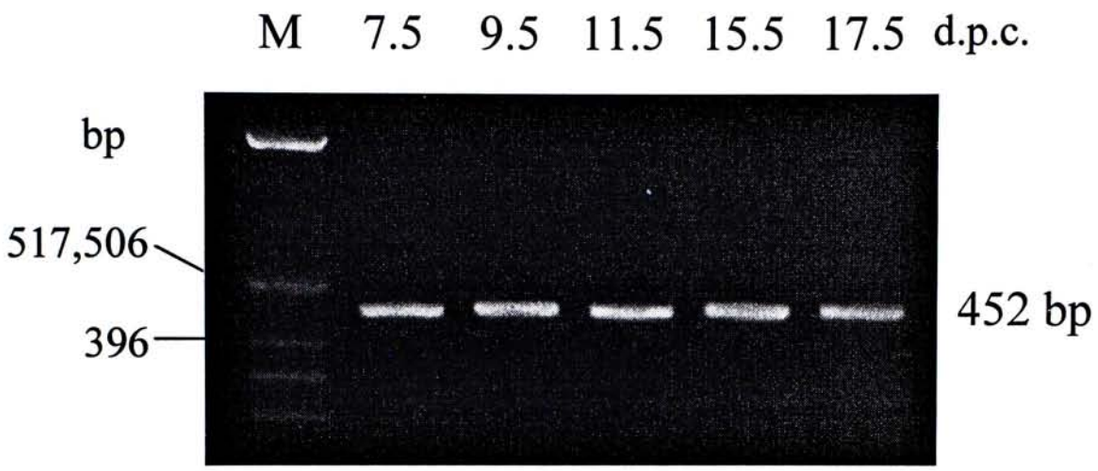


Figure 2.5 Normalization of RT samples by GAPDH gene. RNA of 7.5, 9.5, 11.5, 15.5 and 17.5 d.p.c. embryos were analyzed by RT-PCR. The PCR was performed with primers specific for GAPDH gene for 25 cycles and the products were sequentially resolved in Synergel-agarose gel and stained with EtBr. A fragment of GAPDH gene which was 452 bp in size was generated. Lane **M** is the 1 kb DNA ladder of which 2 marker bands were labeled with their standard sizes on the left-hand-side.

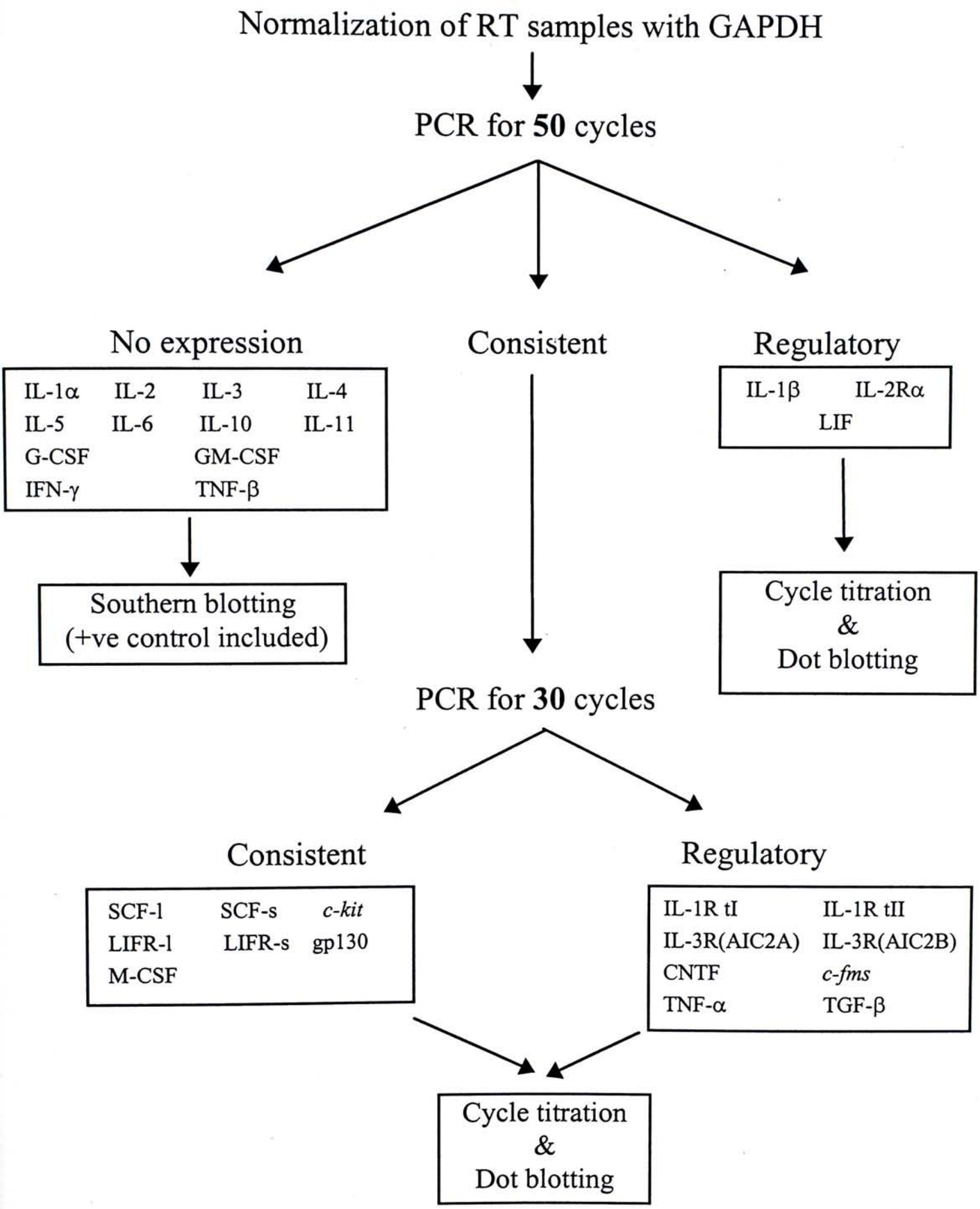


Figure 2.6 Diagram summarizing the result of mRNA phenotyping for 30 cyotkines and cytokine receptors.

The qualitative test discriminated 3 types of cytokines in terms of their expression, they were 'no expression', 'consistent' and 'regulatory' types.

a. Southern hybridization for 'no expression' *cytokines*

'No expression' cytokines included IL-1α, IL-2, IL-3, IL-4, IL-5, IL-6, IL-10, IL-11, GM-CSF, G-CSF, IFN-γ and TNF-β. Their transcripts were detected neither in embryo nor in placenta RNA throughout the 5 stages.

It is essential to include a positive control for the claim of absence of particular transcript. Positive control was cDNA sample previously demonstrated to contain the particular cytokine transcript being amplified with the same pair of primer with the test sample. The control was subjected to PCR in an experiment together with embryo or placenta RT products using the same master mix. It serves to eliminate the possibility of primer problem or the PCR reaction itself. The PCR products were analyzed by Southern hybridization to an internal oligonucleotide. As seen from the X-ray films in **Figure 2.7 - 2.9**, signals were seen only at the positive control bands.

Although IL-1α, IL-2 and IL-3 gene transcripts were absent in mouse embryo or placenta, their receptor IL-1R tI, IL-1R tII, IL-2Rα, IL-3R(AIC2A) and IL-3R(AIC2B) were interestingly expressed. Despite GM-CSF, G-CSF and IL-3 are the major hematopoietic growth factors, they were not detected throughout the embryonic development. IL-6 and IL-11 also intervene hematopoiesis, however, they were also

absent in mouse embryo. These two cytokines both belong to the neuropoietic family which also include LIF, OSM and CNTF. The members share a receptor component gp130 whose transcripts were found in both embryo and placenta tissues.

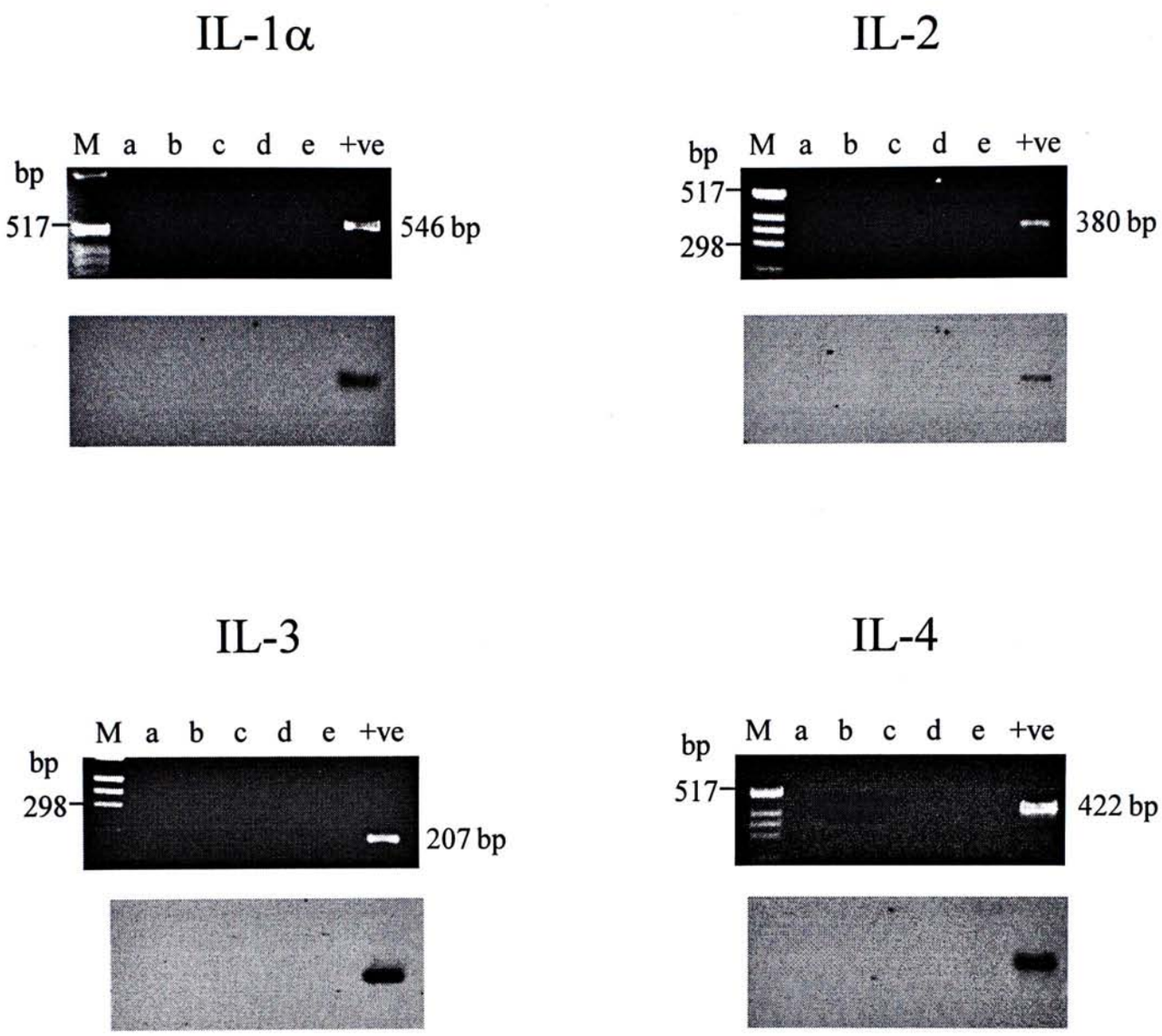


Figure 2.7 Southern hybridization of 'no expression' cytokines IL-1α, IL-2, IL-3 and IL-4. PCR products from embryo tissue as well as positive control (+ve) were electrophoresed on EtBr-stained Synergel-agarose gel and transferred to nylon membrane subsequently. The membrane bound DNAs were then hybridized with corresponding internal probe and signals were recorded on X-ray film (see *Section 2.3.6* for details). Sizes of some marker bands and the corresponding PCR products were marked on the left- and right-hand-side respectively.
M: 1 kb DNA ladder.
(**a**: 7.5 dpc; **b**: 9.5 dpc; **c**: 11.5 dpc; **d**: 15.5 dpc and **e**: 17.5 dpc)

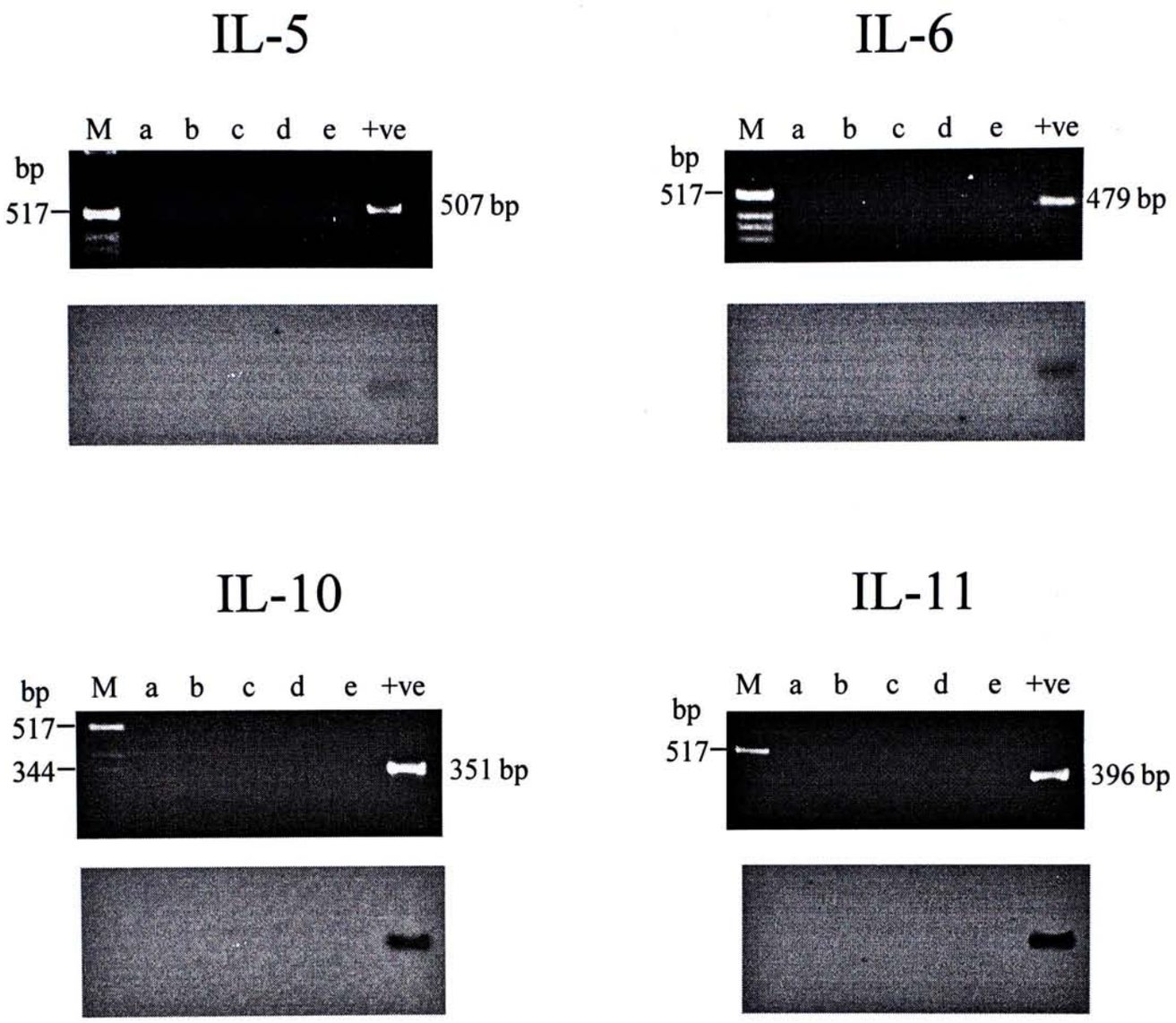


Figure 2.8 Southern hybridization of 'no expression' cytokines IL-5, IL-6, IL-10 and IL-11. PCR products from embryo tissue as well as positive control (+ve) were electrophoresed on EtBr-stained Synergel-agarose gel and transferred to nylon membrane subsequently. The membrane bound DNAs were then hybridized with corresponding internal probe and signals were recorded on X-ray film (see *Section 2.3.6* for details). Sizes of some marker bands and the corresponding PCR products were marked on the left- and right-hand-side respectively.

M: 1 kb DNA ladder.

(**a**: 7.5 dpc; **b**: 9.5 dpc; **c**: 11.5 dpc; **d**: 15.5 dpc and **e**: 17.5 dpc)

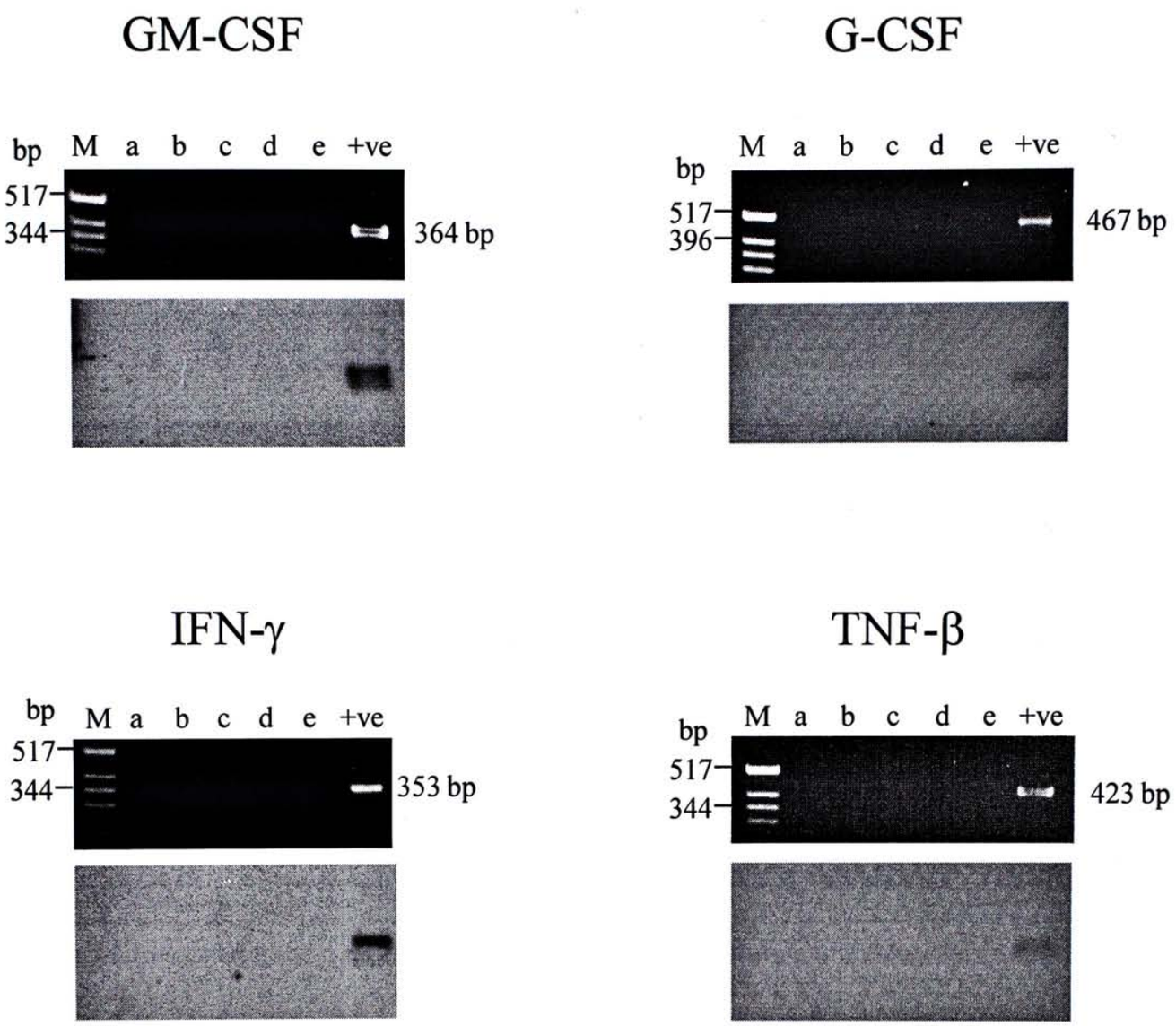


Figure 2.9 Southern hybridization of 'no expression' cytokines GM-CSF, G-CSF, IFN-γ, TNF-β. PCR products from embryo tissue as well as positive control (+ve) were electrophoresed on EtBr-stained Synergel-agarose gel and transferred to nylon membrane subsequently. The membrane bound DNAs were then hybridized with corresponding internal probe and signals were recorded on X-ray film (see *Section 2.3.6* for details). Sizes of some marker bands and the corresponding PCR products were marked on the left- and right-hand-side respectively.

M: 1 kb DNA ladder.

(**a**: 7.5 dpc; **b**: 9.5 dpc; **c**: 11.5 dpc; **d**: 15.5 dpc and **e**: 17.5 dpc)

b. 'Consistent' and 'regulatory' cytokines in embryo and placenta

After 50 cycles of amplification, most cytokines exhibited identical band intensities among the five embryo samples. They were regarded as 'consistent' cytokines. However, they might not truly express at the same level throughout the selected developmental stages. Since the cycle number (50 cycles) was large, the reaction most probably had reached its saturation phase. At this stage, even different levels of initial transcript in different samples would eventually result into same amount of PCR products. Therefore, PCR lowered down to 30 cycles were necessarily performed for these 'consistent' cytokines in the next step.

Once the cycle number was reduced to 30, most 'consistent' cytokine gene turned into 'regulatory', i.e. band intensities varied among different embryonic stages under investigation. These included IL-1R tI and IL-1R tII, IL-3R(AIC2A) and IL-3R(AIC2B), CNTF, *c-fms*, TNF-α and TGF-β. While SCF-l, SCF-s, *c-kit*, M-CSF, LIFR-l, LIFR-s and gp130 still remained at constant levels (**Figure 2.6**).

IL-1β, IL-2Rα and LIF were found to be 'regulatory' at 50 cycles. These cytokines were said to be present in low abundance for the PCR still not reached the plateau phase even after 50 cycles of amplification.

Here, semi-quantitative PCR was adopted to compare the relative amount of cytokine genes in 7.5, 9.5, 11.5, 15.5 and 17.5 d.p.c. mouse embryos and placentas.

PCR products sampled at different cycles were analyzed by dot blotting and probing with an internal probe. As for comparative purpose, the data should be collected during the exponential phase of PCR reaction. The appropriate cycle schedule for each of the cytokines was determined empirically. The band intensity appeared on agarose gel provided certain hints for finding out the suitable cycle numbers.

Dot blotting provides a rapid and convenient method for the analysis of large number of samples. Use of internal oligonucleotide as probe confers an additional level of specificity in the reaction as only the specific PCR product will bind with the probe under the correct stringency of hybridization and washing. The fluorescent signal was exposed on X-ray film for record. Intensity of the signal was proportional to the amount of the homologous sequences to which the probe bound on. Experiments for batch 1 and batch 2 mouse RNA samples were done independently. The results were found to be consistent for the 2 batches of sample and only one set of photos was included here. **Figure 2.10 - 2.14** showed the results for embryo samples while those of placenta samples were shown in **figure 2.15 - 2.19**.

The hybridized dots were scanned with imaging densitometer and their intensities were digitalized as adjusted volume (OD x mm^2). The dot intensity reflected the abundance of PCR product binding to the probe and was used for comparison. **Table 2.3** listed the adjusted volume for cytokines at a particular PCR cycle number where a prominent trend was observed. With regards to dot intensity, the trend of cytokine expression was verbally described in **Table 2.4**.

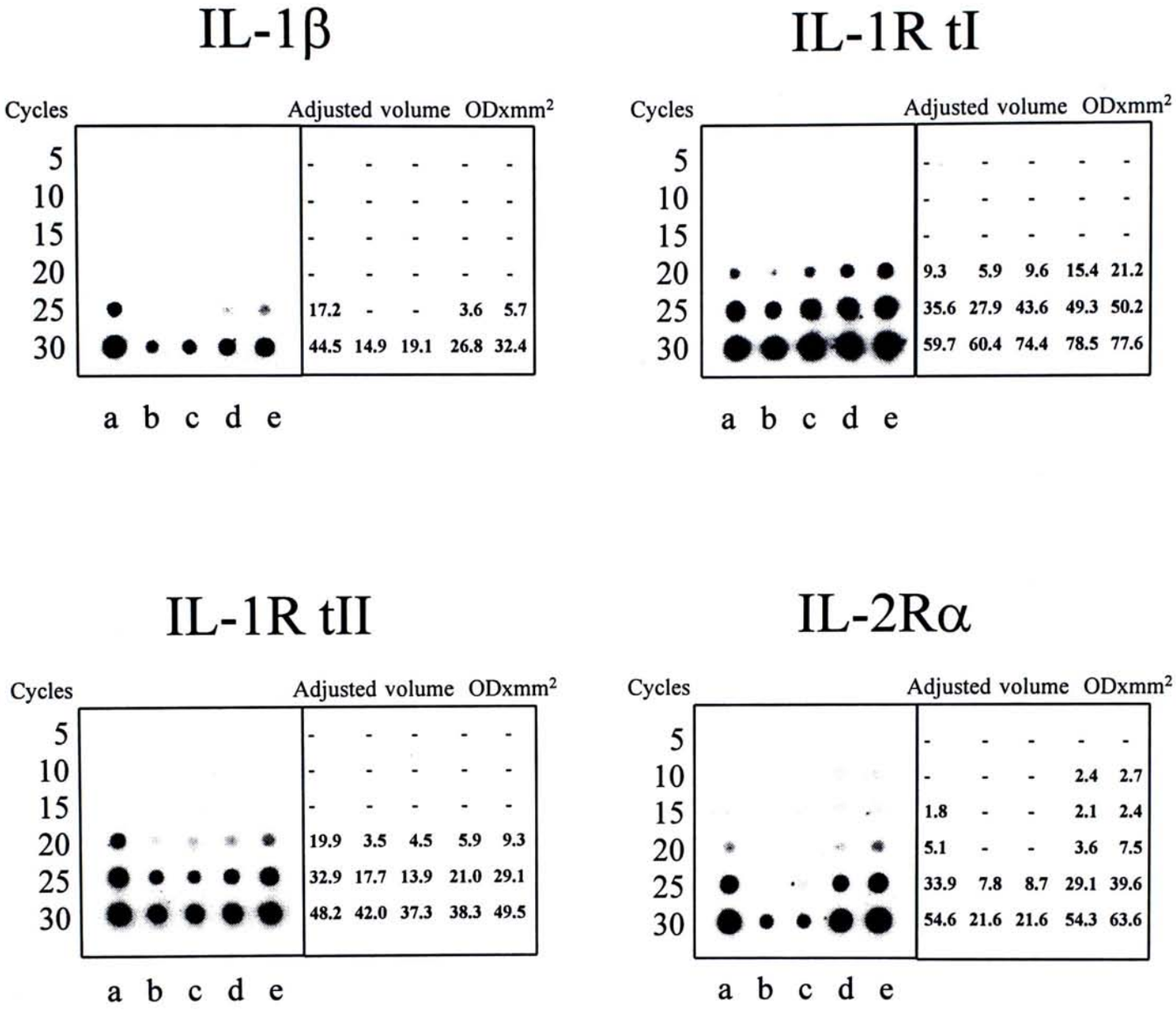
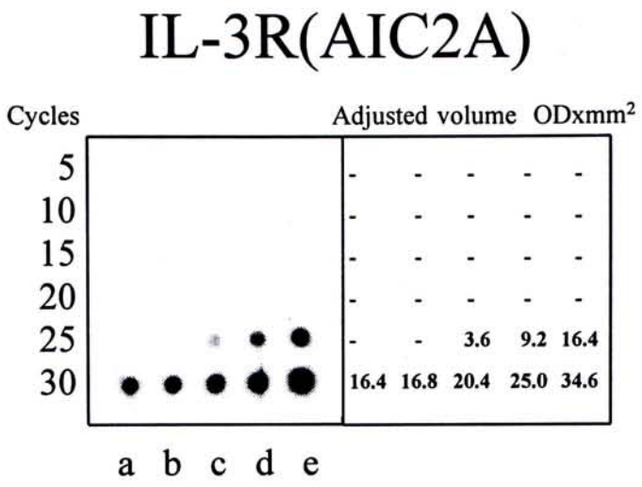


Figure 2.10 Dot-blot hybridization of mouse **embryo** IL-1β, IL-1R tI, IL-1R tII and IL-2Rα RT-PCR products. PCR products of different cycles of amplification (as marked) were dotted on nylon membrane and hybridized with DIG-labeled internal probe. The luminescent signals were recorded on X-ray film (see *Section 2.3.7* for details). The adjusted volume(OD x mm^2) of the dots as determined by imaging densitometer were listed in the box on the right-hand-side.

(**a**: 7.5 dpc; **b**: 9.5 dpc; **c**: 11.5 dpc; **d**: 15.5 dpc; **e**: 17.5 dpc)



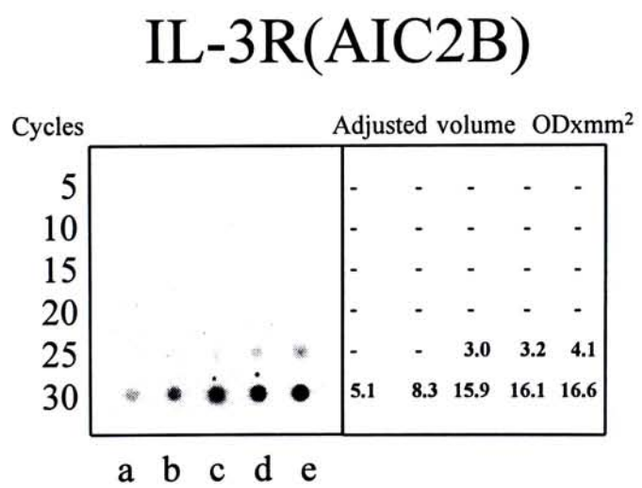


Figure 2.11 Dot-blot hybridization of mouse **embryo** IL-3R(AIC2A) and IL-3R(AIC2B) RT-PCR products. PCR products of different cycles of amplification (as marked) were dotted on nylon membrane and hybridized with DIG-labeled internal probe. The luminescent signals were recorded on X-ray film (see *Section 2.3.7* for details). The adjusted volume (OD x mm^2) of the dots as determined by imaging densitometer were listed in the box on the right-hand-side.

(**a**: 7.5 dpc; **b**: 9.5 dpc; **c**: 11.5 dpc; **d**: 15.5 dpc; **e**: 17.5 dpc)

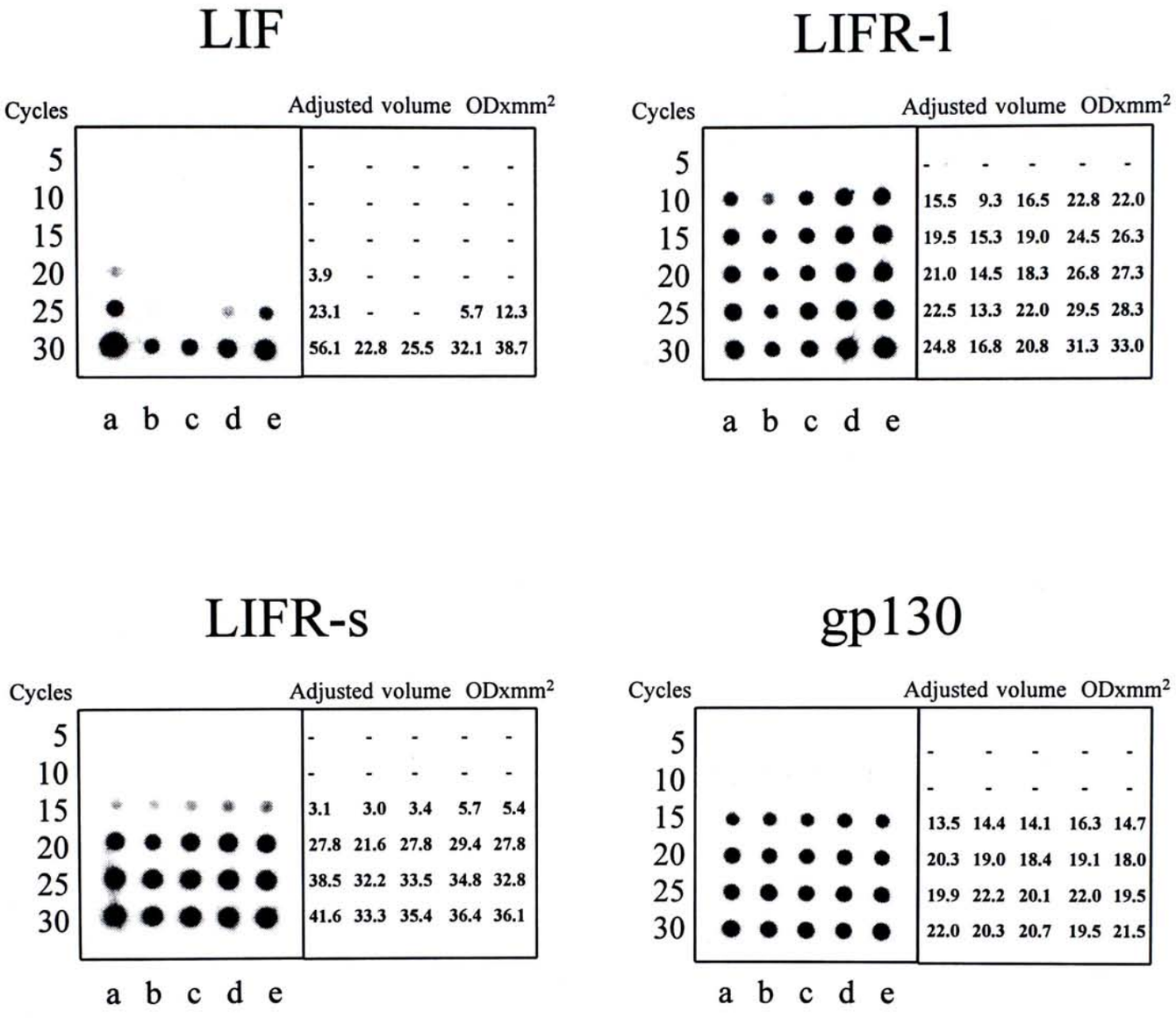


Figure 2.12 Dot-blot hybridization of mouse **embryo** LIF, LIFR-l, LIFR-s and gp130 RT-PCR products. PCR products of different cycles of amplification (as marked) were dotted on nylon membrane and hybridized with DIG-labeled internal probe. The luminescent signals were recorded on X-ray film (see *Section 2.3.7* for details). The adjusted volume (OD x mm^2) of the dots as determined by imaging densitometer were listed in the box on the right-hand-side.

(**a**: 7.5 dpc; **b**: 9.5 dpc; **c**: 11.5 dpc; **d**: 15.5 dpc; **e**: 17.5 dpc)

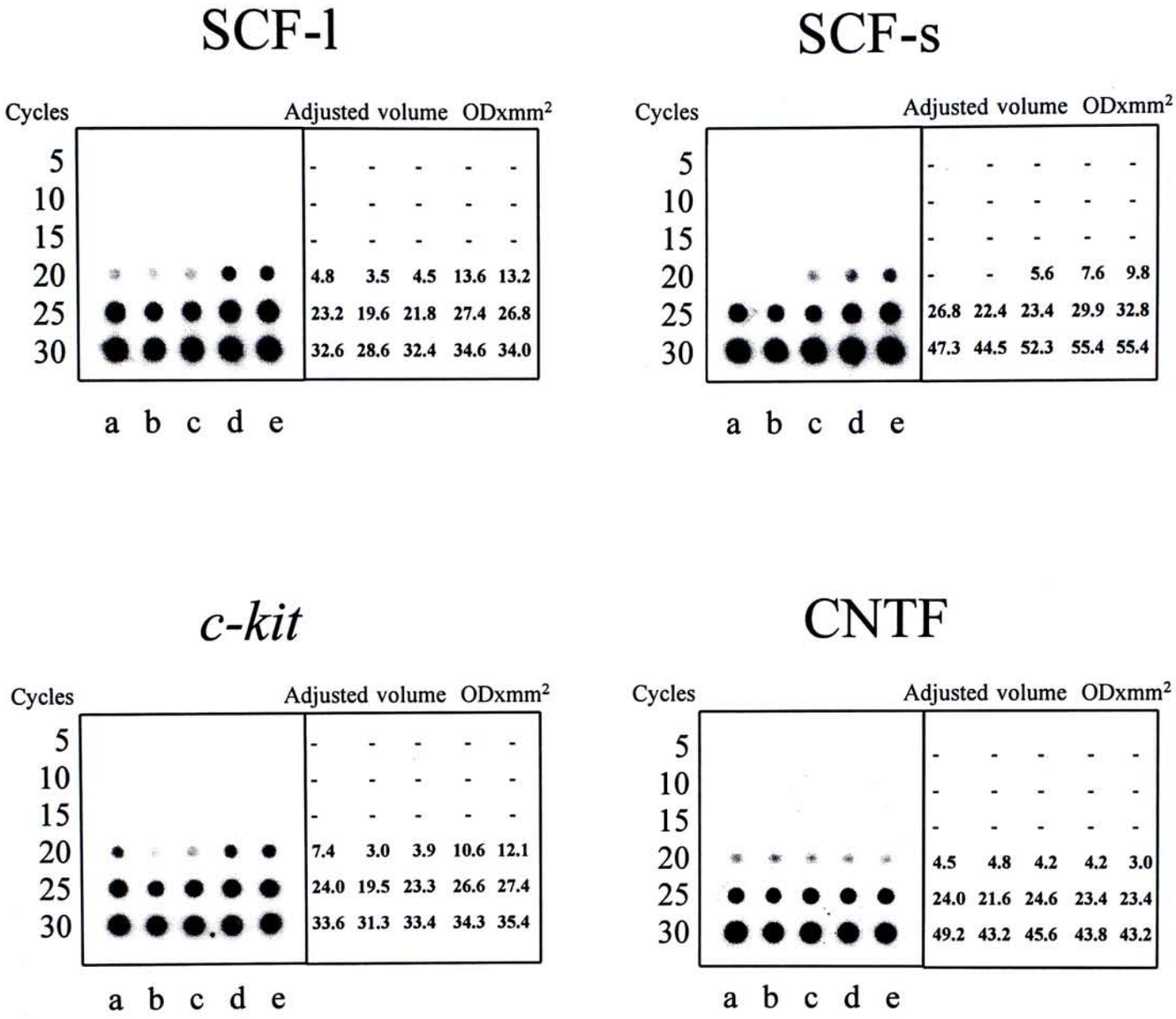


Figure 2.13 Dot-blot hybridization of mouse **embryo** SCF-l, SCF-s, *c-kit* and CNTF RT-PCR products. PCR products of different cycles of amplification (as marked) were dotted on nylon membrane and hybridized with DIG-labeled internal probe. The luminescent signals were recorded on X-ray film (see *Section 2.3.7* for details). The adjusted volume (OD x mm²) of the dots as determined by imaging densitometer were listed in the box on the right-hand-side.

(**a**: 7.5 dpc; **b**: 9.5 dpc; **c**: 11.5 dpc; **d**: 15.5 dpc; **e**: 17.5 dpc)

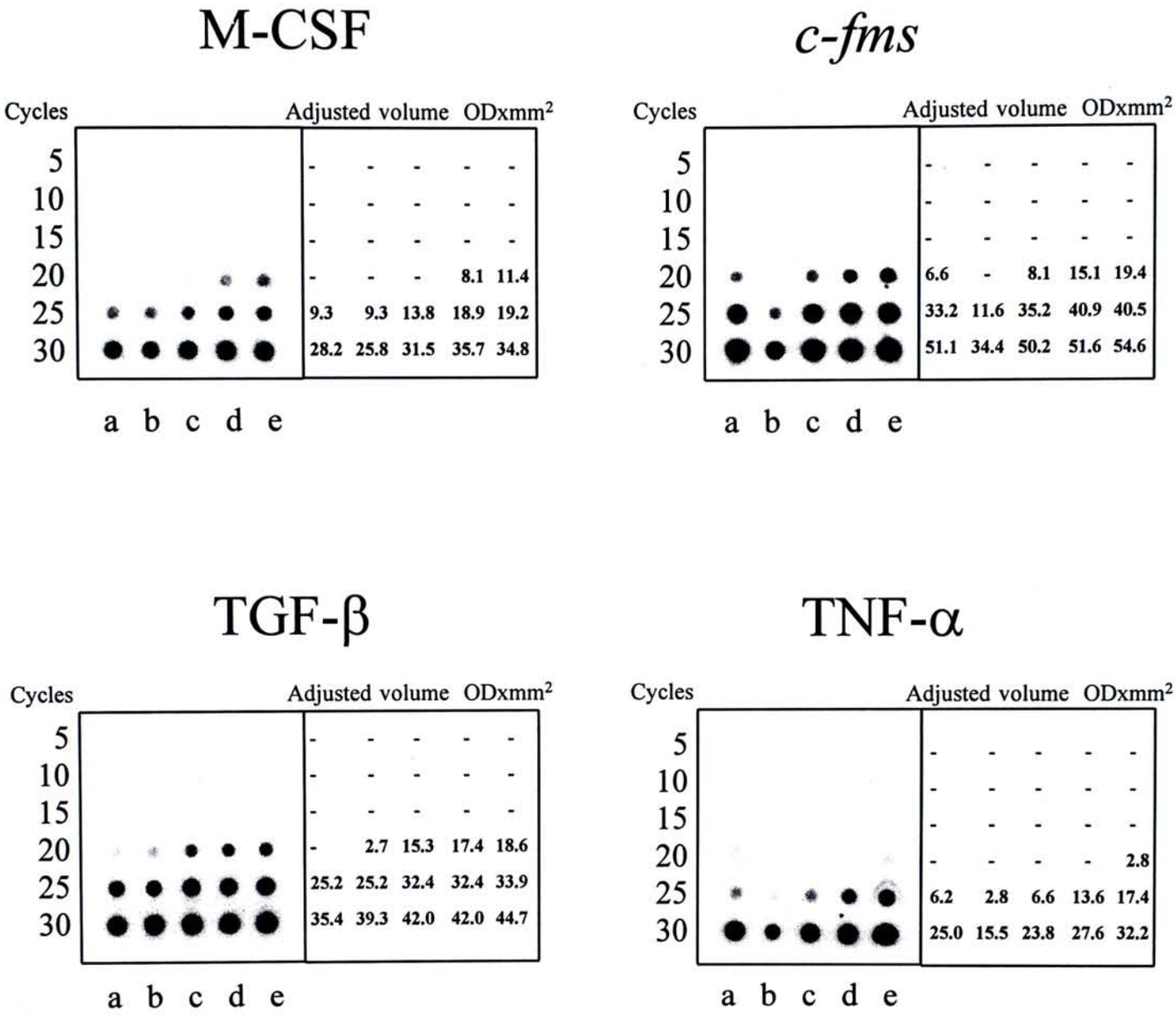


Figure 2.14 Dot-blot hybridization of mouse **embryo** M-CSF, *c-fms*, TGF-β and TNF-α RT-PCR products. PCR products of different cycles of amplification (as marked) were dotted on nylon membrane and hybridized with DIG-labeled internal probe. The luminescent signals were recorded on X-ray film (see *Section 2.3.7* for details). The adjusted volume(OD x mm²) of the dots as determined by imaging densitometer were listed in the box on the right-hand-side.

(**a**: 7.5 dpc; **b**: 9.5 dpc; **c**: 11.5 dpc; **d**: 15.5 dpc; **e**: 17.5 dpc)

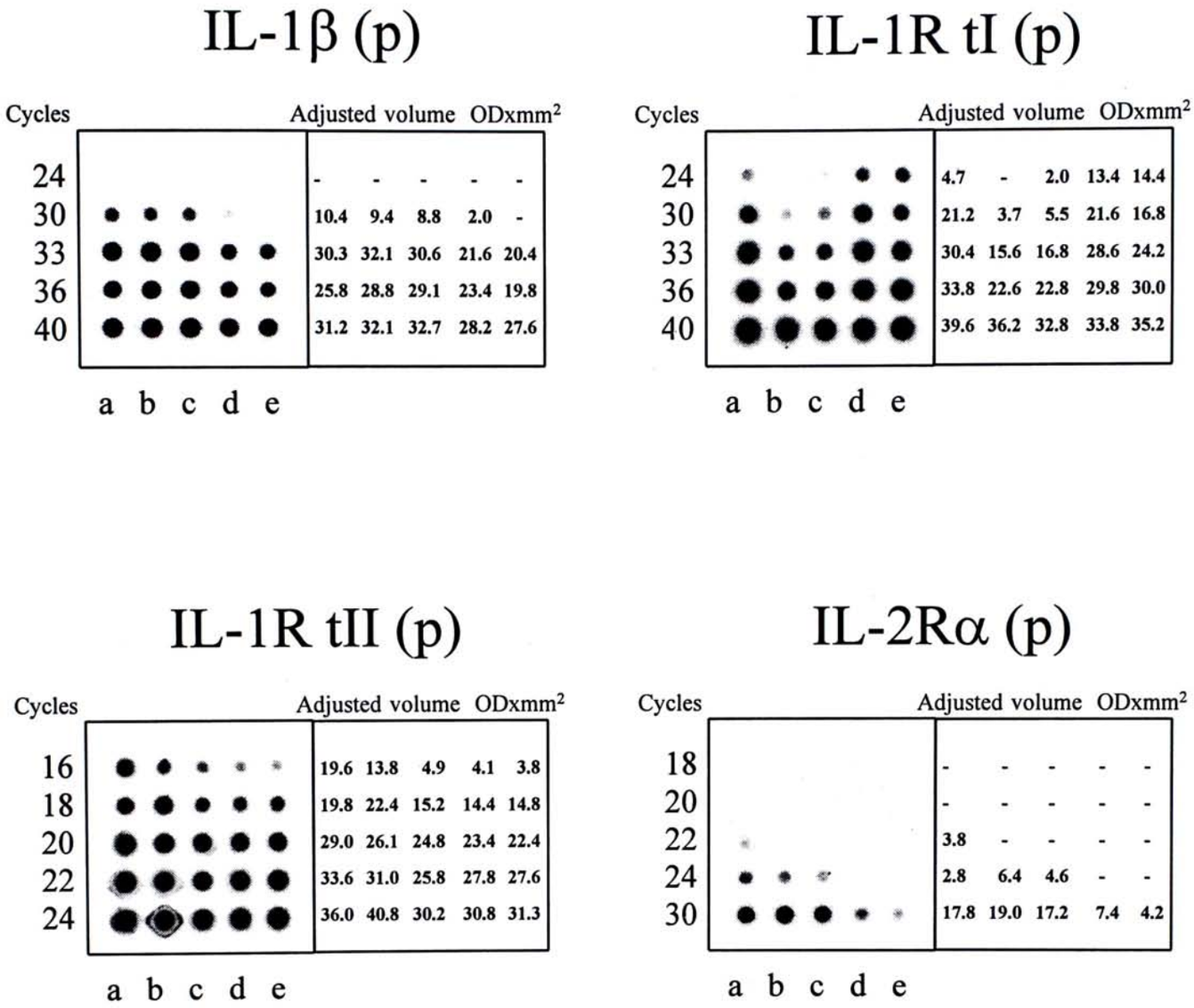
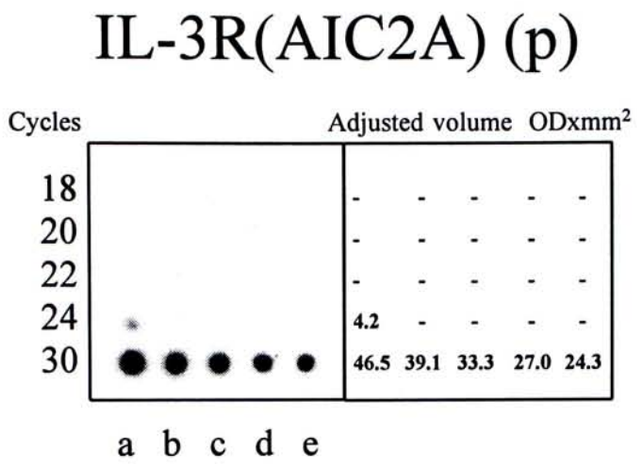


Figure 2.15 Dot-blot hybridization of mouse **placental** IL-1β, IL-1R tI, IL-1R tII and IL-2Rα RT-PCR products. PCR products of different cycles of amplification (as marked) were dotted on nylon membrane and hybridized with DIG-labeled internal probe. The luminescent signals were recorded on X-ray film (see *Section 2.3.7* for details). The adjusted volume(OD x mm²) of the dots as determined by imaging densitometer were listed in the box on the right-hand-side. **(p)** denotes placental tissue.
(**a**: 7.5 dpc; **b**: 9.5 dpc; **c**: 11.5 dpc; **d**: 15.5 dpc; **e**: 17.5 dpc)



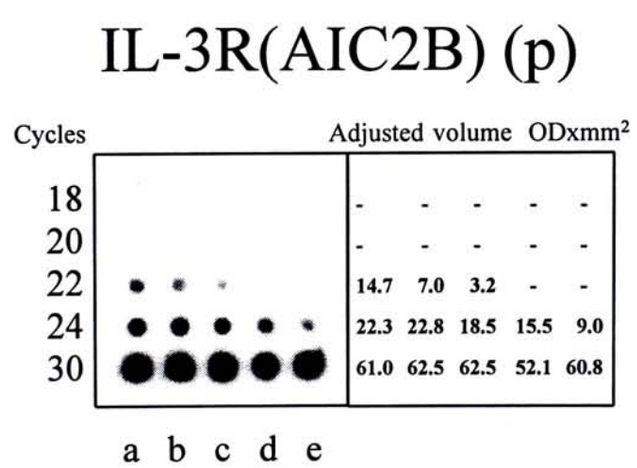


Figure 2.16 Dot-blot hybridization of mouse **placental** IL-3R(AIC2A) and IL-3R(AIC2B) RT-PCR products. PCR products of different cycles of amplification (as marked) were dotted on nylon membrane and hybridized with DIG-labeled internal probe. The luminescent signals were recorded on X-ray film (see *Section 2.3.7* for details). The adjusted volume(OD x mm^2) of the dots as determined by imaging densitometer were listed in the box on the right-hand-side. **(p)** denotes placental tissue.
(**a**: 7.5 dpc; **b**: 9.5 dpc; **c**: 11.5 dpc; **d**: 15.5 dpc; **e**: 17.5 dpc)

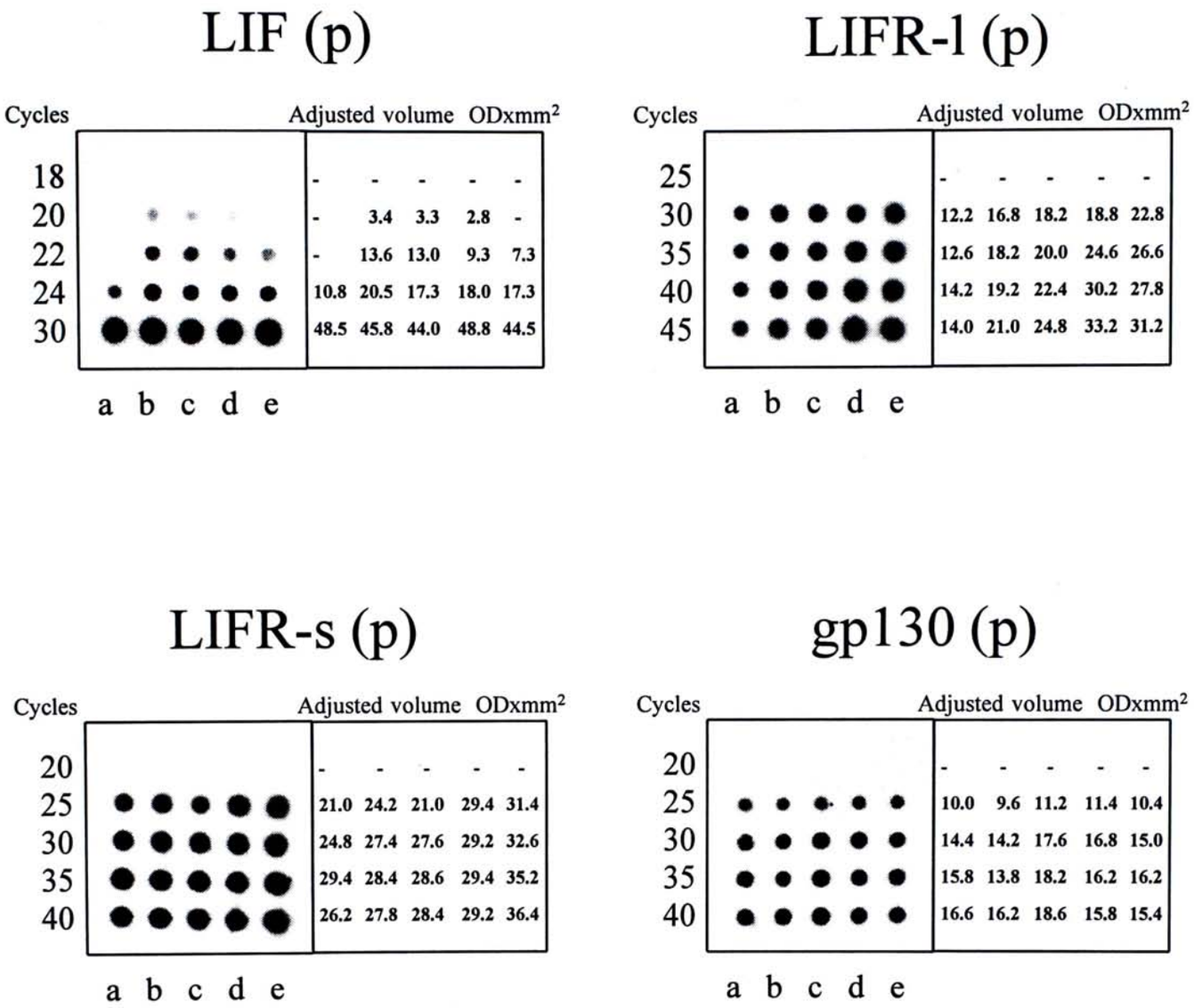


Figure 2.17 Dot-blot hybridization of mouse **placental** LIF, LIFR-l, LIFR-s and gp130 RT-PCR products. PCR products of different cycles of amplification (as marked) were dotted on nylon membrane and hybridized with DIG-labeled internal probe. The luminescent signals were recorded on X-ray film (see *Section 2.3.7* for details). The adjusted volume(OD x mm^2) of the dots as determined by imaging densitometer were listed in the box on the right-hand-side. **(p)** denotes placental tissue.
(**a**: 7.5 dpc; **b**: 9.5 dpc; **c**: 11.5 dpc; **d**: 15.5 dpc; **e**: 17.5 dpc)

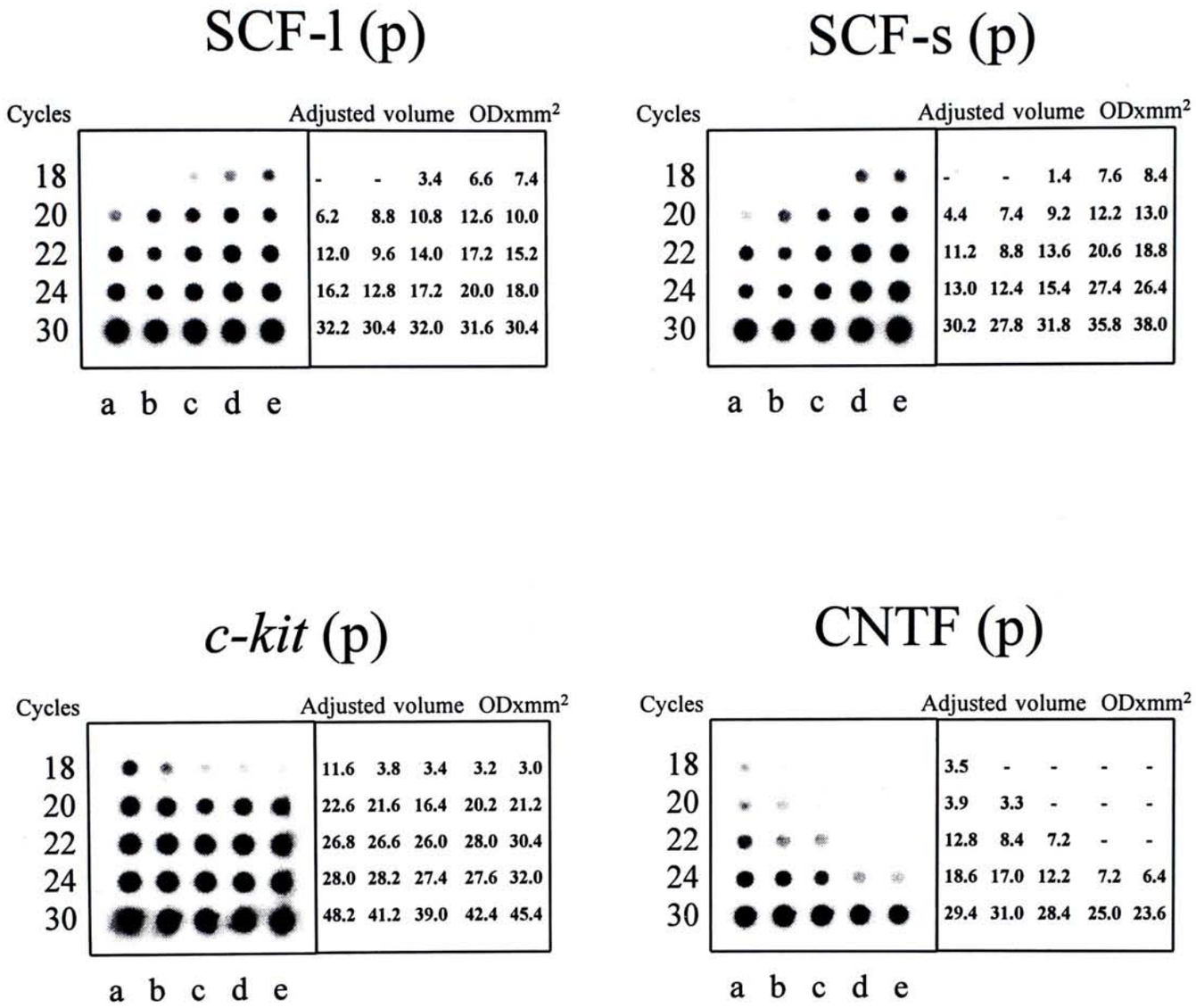


Figure 2.18 Dot-blot hybridization of mouse **placental** SCF-l, SCF-s, *c-kit* and CNTF RT-PCR products. PCR products of different cycles of amplification (as marked) were dotted on nylon membrane and hybridized with DIG-labeled internal probe. The luminescent signals were recorded on X-ray film (see *Section 2.3.7* for details). The adjusted volume(OD x mm^2) of the dots as determined by imaging densitometer were listed in the box on the right-hand-side. **(p)** denotes placental tissue.
(**a**: 7.5 dpc; **b**: 9.5 dpc; **c**: 11.5 dpc; **d**: 15.5 dpc; **e**: 17.5 dpc)

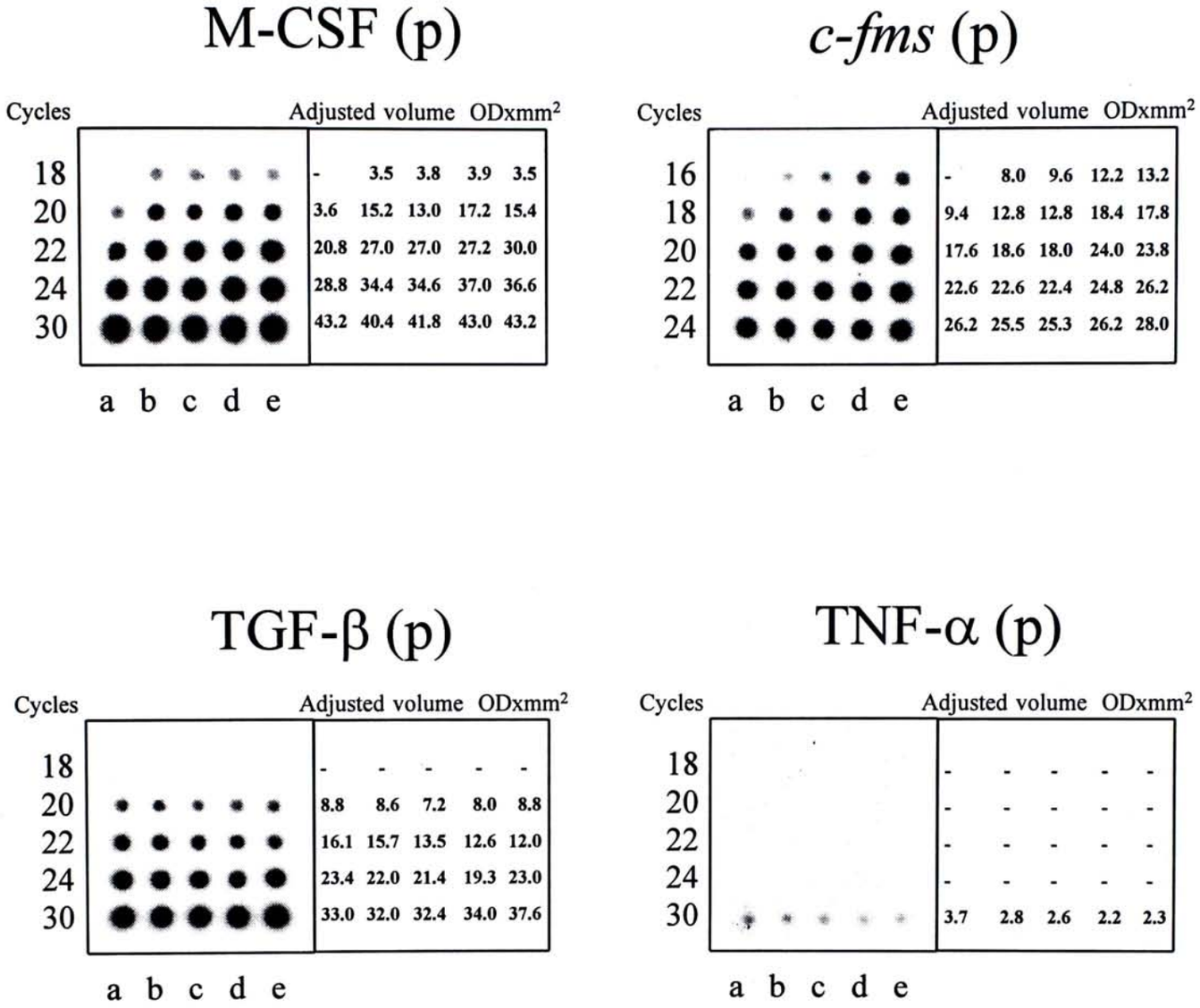


Figure 2.19 Dot-blot hybridization of mouse **placental** M-CSF, *c-fms*, TGF-β and TNF-α RT-PCR products. PCR products of different cycles of amplification (as marked) were dotted on nylon membrane and hybridized with DIG-labeled internal probe. The luminescent signals were recorded on X-ray film (see *Section 2.3.7* for details). The adjusted volume(OD x mm^2) of the dots as determined by imaging densitometer were listed in the box on the right-hand-side. **(p)** denotes placental tissue.
(**a**: 7.5 dpc; **b**: 9.5 dpc; **c**: 11.5 dpc; **d**: 15.5 dpc; **e**: 17.5 dpc)

No.	Gene	Embryo (d.p.c.)					Placenta (d.p.c.)				
		7.5	9.5	11.5	15.5	17.5	7.5	9.5	11.5	15.5	17.5
1	IL-1β	44.5	14.9	19.1	26.8	32.4	30.3	32.1	30.6	21.6	20.4
2	IL-1R tI	9.3	5.9	9.6	15.4	21.2	21.2	3.7	5.5	21.6	16.8
3	IL-1R tII	32.9	17.7	13.9	21.0	29.1	19.6	13.8	4.9	4.1	3.8
4	IL-2Rα	54.6	21.6	21.6	54.3	63.6	17.8	19.0	17.2	7.4	4.2
5	IL-3R(AIC2A)	16.4	16.8	20.4	25.0	34.6	46.5	39.1	33.3	27.0	24.3
6	IL-3R(AIC2B)	5.1	8.3	15.9	16.1	16.6	22.3	22.8	18.5	15.5	9.0
7	LIF	56.1	22.8	25.5	32.1	38.7	10.8	20.5	17.3	18.0	17.3
8	LIFR-l	15.5	9.3	16.5	22.8	22.0	12.2	16.8	18.2	18.8	22.8
9	LIFR-s	27.8	21.6	27.8	29.4	27.8	24.8	27.4	27.6	29.2	32.6
10	gp130	13.5	14.4	14.1	16.3	14.7	10.0	9.6	11.2	11.4	10.4
11	SCF-l	23.2	19.6	21.8	27.4	26.8	6.2	8.8	10.8	12.6	10.0
12	SCF-s	26.8	22.4	23.4	29.9	32.8	4.4	7.4	9.2	10.2	13.0
13	*c-kit*	7.4	3.0	3.9	10.6	12.1	11.6	3.8	3.4	3.2	3.0
14	CNTF	24.0	21.6	24.6	23.4	23.4	18.6	17.0	12.2	7.2	6.4
15	M-CSF	9.3	9.3	13.8	18.9	19.2	3.6	15.2	13.0	17.2	15.4
16	*c-fms*	33.2	11.6	35.2	40.9	40.5	9.4	12.8	12.8	18.4	17.8
17	TGF-β	25.2	25.2	32.4	32.4	33.9	8.6	8.6	7.2	8.0	8.8
18	TNF-α	6.2	2.8	6.6	13.6	17.4	3.7	2.8	2.6	2.2	2.3

Table 2.3 The adjusted volume (OD x mm^2) of hybridization signal dots at a particular PCR cycle number where prominent trend was observed (before saturation phase of PCR).

The dot blotting results were summarized in the following table:

No.	Gene	Embryo (7.5 - 17.5 d.p.c.)	Placenta (7.5 - 17.5 d.p.c.)
1	**IL-1β**	down-regulated from 7.5 onwards	*gradually decreased*
2	**IL-1R tI**	up-regulated from 9.5 onwards	down-regulated at 9.5 and 11.5
3	**IL-1R tII**	down-regulated from 7.5 onwards	*gradually decreased*
4	**IL-2Rα**	down-regulated at 9.5 and 11.5	*gradually decreased*
5	**IL-3R(AIC2A)**	gradually increased	*gradually decreased*
6	**IL-3R(AIC2B)**	gradually increased	*gradually decreased*
7	**LIF**	down-regulated from 7.5 onwards	up-regulated at 9.5, then decreased
8	**LIFR-l**	fairly constant, but weak at 9.5	mildly increased
9	**LIFR-s**	fairly constant, but weak at 9.5	fairly constant
10	**gp130**	fairly constant	fairly constant
11	**SCF-l**	gradually increased	gradually increased
12	**SCF-s**	gradually increased	gradually increased
13	***c-kit***	down-regulated at 9.5,then increased	*gradually decreased*
14	**CNTF**	fairly constant	*gradually decreased*
15	**M-CSF**	gradually increased	constant but weak at 7.5
16	***c-fms***	down-regulated at 9.5,then increased	gradually increased
17	**TGF-β**	gradually increased	fairly constant
18	**TNF-α**	down-regulated at 9.5,then increased	fairly constant

Table 2.4 The pattern of cytokine expression in embryo and placenta from 7.5 to 17.5 d.p.c. as summarized from the results of dot-blot hybridization **(Figure 2.10-2.19)**.

c. The expression pattern in embryo vs placenta

Both IL-1α and IL-1β will bind to IL-1R tI and IL-1R tII, however, IL-1α was not found in all stages of embryos under investigation. Whereas IL-1β was detected to be down-regulated from 7.5 d.p.c. onwards. Type II IL-1R had the same pattern with IL-1β while type I receptor conversely showed an up-regulation from 9.5 to 17.5 d.p.c..

In placenta, IL-1β and IL-1R tII also down-regulated as in embryo tissue. However, IL-1R tI was up-regulated at 15.5 and 17.5 d.p.c. but down-regulated at 9.5 and 11.5 d.p.c..

Although IL-2 and IL-3 were absent from all embryonic tissue, their receptors were interestingly present. IL-2Rα was specially down-regulated in 9.5 and 11.5 d.p.c. among the 5 time-points. The gene transcripts of IL-3R(AIC2A) and IL-3R(AIC2B) showed a gradual increase as the embryo grew from 7.5 to 17.5 d.p.c..

In placenta, there was a descending trend of IL-2Rα expression. The patterns of IL-3R(AIC2A) and IL-3R(AIC2B) in embryo and in placenta were just opposite. The amount of the two receptor subunits increased in embryo while decreased in placenta as time of gestation grew.

LIF which can inhibit embryonic stem cell differentiation was down-regulated in embryos from 7.5 d.p.c. onwards, especially at 9.5 and 11.5 d.p.c.. The levels of its receptors, both soluble and transmembrane forms, were fairly constant among the 5 points except a small drop at 9.5 d.p.c.. The signal transduction subunit for LIF, gp130,

displayed a consistent level throughout the developmental stages. Although CNTF is grouped under the same cytokine subfamily with LIF, it did not demonstrate down-regulation but expressed at constant level instead.

In placenta, there was a up flush of LIF at 9.5 d.p.c. and then the amount decreased onwards. Similar to the trend in embryo, the level of LIFR-l, LIFR-s and gp130 were fairly constant. CNTF, however, exhibited a decrease in placental tissue.

In embryo, the amount of SCF-l, SCF-s and M-CSF transcripts increased from 7.5 to 17.5 d.p.c.. However, their respective receptor, *c-kit* and *c-fms* did not exhibit similar ascending trend but down-regulated specially at time point 9.5 d.p.c.. Like SCF and M-CSF, TGF-β and TNF-α also up-regulated from 9.5 d.p.c. onwards.

In placenta, both SCF-l and SCF-s levels also showed a gradual increase, similar to the trend in embryo. For *c-kit*, its level gradually decreased from 7.5 to 17.5 d.p.c.. The amount of M-CSF, TGF-β and TNF-α transcripts remained fairly constant in placenta. It is worth to note that the expression level of TNF-α mRNA was very low in the placental tissue when compared with that in the embryo.

2.5 Discussion

2.5.1 Isolation of embryo RNA by guanidinium thiocyanate/ cesium chloride centrifugation

Many methodologies have been empirically derived to accomplish the expedient isolation of RNA. The most common techniques include the use of guanidinium buffers, phenol, SDS, ethanol precipitation or CsCl gradient centrifugation. No matter which approach is chosen, three criteria have to be achieved in order to purify high quality and undegraded RNA from cells and tissues. They are (1) inhibition of endogenous nucleases, (2) deproteinization of the RNA, and (3) physical separation of the RNA from the other components of the homogenate (MacDonald *et al.*, 1987).

In the present project, guanidinium thiocyanate / cesium chloride centrifugation is the method of choice for preparing embryo RNA. Guanidinium thiocyanate (GT), which is a strong protein denaturant, not only effectively deproteinize sample tissues but also eliminate nucleolytic activities. Due to the extremely chaotropic nature of GT, it is the best way to deal with recalcitrant RNases with GT lysis buffer. In the cases of intractable tissues, or very dilute RNA solutions, ultracentrifugation through CsCl gradient to separate RNA from other components is recommended for quantitative recovery of RNA. To do a detailed screening of cytokine expression profile, large amount of RNA have to be prepared. Therefore, the choice of protocol for RNA preparation is important here.

Mouse embryos at different stages vary in their size. For 7.5 d.p.c. embryo, they are so tiny that only limited quantity of RNA is available from each individual. On the contrary, older embryos (15.5 and 17.5 d.p.c.) are large enough to provide sufficient RNA, but the developed embryos contain lots of intractable tissues such as ligament and cartilage. For these reasons, an effective method for RNA isolation is essential. Although GT/ CsCl gradient requires an overnight centrifugation in an ultracentrifuge (18 hrs), it is still worthwhile to adopt this approach which reproducibly yields the highest quality RNA sample.

In order to increase the accuracy of the result, two batches of embryo RNAs were taken from two separate groups of pregnant mice. All the experimental procedures were repeated for the second batch of mouse embryo RNA of each selected stage. Consistency of the data collected from the two batches of sample RNA in two independent experiments can diminish the possibility of experimental error.

2.5.2 mRNA Quantitation

Since cytokines regulate cell growth and differentiation, their expression level may change as the embryo grows. In order to figure out a picture of cytokine expression profile along the time axis of mouse development, the amount of interested cytokine mRNA in each embryonic stage has to be determined. Northern blot, S1 nucleases assays, RNase protection and *in situ* hybridization methods are currently

used for analyzing RNA levels, however, they require large quantities of RNA and are not very sensitive for quantitative use in detecting low abundance mRNA. Furthermore, these methods are time consuming, technically difficult and not suitable for a simultaneous processing of a large number of samples.

PCR has been adapted to amplify specific mRNA sequences after a first step of reverse transcription. This not only permits the detection of low abundance mRNAs, but also the development of a number of quantitative procedures for the analysis of RNA levels (Chelly and Kahn, 1994).

Quantitative PCR is made complicated by two inherent features of *in vitro* amplification. One is the exponential phase of reaction in which minute differences in variables (e.g. concentration of enzyme, templates, primers) can greatly influence the PCR product yield. The other feature of PCR is the plateau phase where the reaction rate declines dramatically as a consequence of reaction component consumption and generation of inhibitors such as pyrophosphates, final PCR products (Köhler, 1995; Siebert, 1993). Therefore, most researchers use internal standards to eliminate tube-to-tube variations and to determine absolute values of mRNA.

Two types of internal standard can be used. One is endogenous sequence or gene transcript known to be present at constant levels throughout a series of samples. They are typically housekeeping gene, such as β-actin (Kinoshita *et al.*, 1994), β_2-microglobulin (Murphy *et al.*, 1990) and GAPDH (Scheuermann and Bauer, 1993). The other type is an exogenous fragment added to the target sample and amplified

simultaneously with the target transcript in a single PCR reaction mixture. These standards that share the same primer annealing sequences with the target allow calculation of the absolute amount of target mRNA, as described by Wang *et al.* (1989). Each type of internal control has advantages and limitations. However, most problems can be circumvented by using competitive PCR in which an exogenous template having identical primer binding sites as target is co-amplified in a competitive fashion (Siebert, 1993).

Semi-quantitative PCR

In many situations, it is sufficient to compare the relative amounts of a particular target sequence in different samples but not necessary to determine the actual number of starting target molecules. A simpler approach named as 'semi-quantitative PCR' can be adopted for this purpose (Dallman and Porter, 1991). The essence of this approach is to sample product from PCR at multiple points throughout the amplification process. It is actually a kinetic analysis in which the amount of amplified products are plotted against cycle numbers. If the data are collected before the plateau phase is reached, the difference in the amount of amplified products is equal to the difference in the abundance of that particular transcript in the starting materials.

The aim of this part of thesis is to determine the expression pattern of various cytokine genes during embryonic development. It is, therefore, not necessary to work out the exact number of interested mRNA. Semi-quantitative PCR is sufficient to

accomplish this objective. A comparison of the level of cytokine mRNA in different embryonic stages (7.5, 9.5, 11.5, 15.5 and 17.5 d.p.c.) has been performed using this approach. This method has the advantage of simplicity and is readily be applied to any previously defined PCR conditions making it easily accessible to the analysis of different target sequences.

2.5.3 Cytokine mRNA phenotyping by RT-PCR

a. Reverse Transcription

Reverse transcription (RT) might be primed by using a specific antisense primer for the gene of interest, random hexanucleotide primers, or oligo d(T). The latter approach is most consistent and results in the highest amplification of target sequence. Besides, the use of Oligo d(T) in cDNA synthesis also produces same sample for the amplification of any desired target transcripts. Therefore, Oligo $d(T)_{12-18}$ was chosen as the RT primer in the present project.

For the relative quantitation method, cDNAs to be compared after PCR were synthesized in parallel using the same reaction mix, so as to improve the accuracy.

b. GAPDH as a control for normalization

In all the RT-PCR experiment, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was regularly used to judge the success of RNA preparations with regard to degradation of RNA and efficacy of reverse transcription. To perform relative quantitation of specific transcripts, the prerequisite is to demonstrate that each sample has undergone equivalent reverse transcription to cDNA and equivalent amplification in the PCR, in other words, to normalize all the samples. GAPDH is a metabolic enzyme known to be present at constant level in various cell types. Normalization was therefore done by using a pair of primer specific to this enzyme gene transcript. Samples were said to be normalized when there was no greater than 2-fold difference in the band intensity of GAPDH PCR product among the 5 embryonic RNA samples, as resolved on agarose gel. Comparative data of cytokine gene expression was collected and validated only after the RT samples were equalized by GAPDH control. Since the control sequence is not being used to define absolute levels of mRNA, differences in primer efficiencies between the control and test primers are not important.

Figure 2.5 shows one of the gel photos in which the intensities of GAPDH bands amplified from the 5 RNA samples did not have more than 2-fold difference from each other. Regularly, normalization was performed by subjecting equal amount of RT product from each embryonic stage to PCR for 25 cycles where the reaction had not reached the plateau phase yet. In cases when difference in the band intensity was detected, volume of RT for PCR were adjusted accordingly so as to attain

normalization among the 5 samples. GAPDH control was always included in all the experiments of comparative purpose, in order to eliminate day-to-day and tube-to-tube variations.

c. PCR for cytokine transcripts

One prerequisite for the detection of gene transcripts by PCR method is the use of a pair of specific and efficient primer. Most literatured and well studied cytokine genes in mouse have already been sequenced and the sequence information is available from special databases via data networks. With the sequences on hand, primer search was started with the aid of an automated primer search software, OLIGO 3.4 Primer Analysis Software. Although there is a general set of rules for primer design (as listed in *Section 2.1.2*), it cannot ensure the synthesis of an effective primer pair. As a matter of fact, the selection is somewhat empirical. Fortunately, the primers used in this project were tested to work satisfactorily. They were designed to be situated on two different exons and spanning at least one intron to allow unambiguous discrimination between cDNA and unwelcome genomic amplification products. The products were further analyzed by hybridizing with an internal probe that lies within the target sequence between the two original primers.

Thirty genes of cytokines and cytokine receptors were studied for their pattern of expression in both the embryo and the placenta during development from 7.5 to 17.5 d.p.c. The first step was to perform a qualitative test to check the presence of

transcripts. It was done by 50 cycles of PCR using specific primers. After this number of amplification cycles, even low abundance RNA could easily be detected on the agarose gel. It was always established by gel electrophoresis that under the conditions used the PCR resulted in a single product of the expected size. No banding means the absence of that particular transcript in the RNA sample.

2.5.4 Cytokines and receptors in embryonic development

During embryogenesis, cell-to-cell interactions are one of the principal mechanisms by which differentiation, morphogenesis, and growth are regulated. As cytokines are associated with intercellular communication, these molecules must play crucial roles in mastering the development of embryos. In order to determine the *in vivo* embryonic role of cytokines, the first step is to determine when and where they are expressed during the life cycle of the mouse. In the first part of the thesis, the expression patterns of 30 cytokines and cytokine receptors during mid- to late-gestation (7.5 to 17.5 d.p.c.) of ICR mouse were investigated using reverse transcription and semi-quantitative PCR.

RT-PCR allows detection of transcripts of low copy number in embryos, obviating the need to obtain large amounts of embryonic tissue required for standard RNA analysis. Since it was not necessary to work out the exact amount of cytokine transcripts, a simpler and time-saving method, semi-quantitative PCR, was adopted to determine the relative levels of cytokine mRNA in different stages of mouse embryo. The results revealed that more than half of the investigated cytokines and receptors had regulatory patterns among the 5 developmental stages, underlying certain biological functions in regulating proper embryonic development.

2.5.4.1 Cytokines in hematopoietic development of mouse fetus

In mouse, the formation of blood cells, or hematopoiesis, begins in the yolk sac at approximately day 7 of embryogenesis. The major site of hematopoiesis shifts to the fetal liver by day 10 of gestation and finally to the bone marrow, which remains the major hematopoietic organ in the adult (Moore and Metcalf, 1970).

Some cytokines have been shown to be important to hematopoietic development, such as IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, GM-CSF, G-CSF, M-CSF and its receptor *c-fms*, SCF and its receptor *c-kit* (Nakayama *et al.*, 1989; Murray *et al.*, 1990; Schmitt *et al.*, 1991; Heike *et al.*, 1994). These factors range from being lineage specific, such as M-CSF (Stanley and Heard, 1977) and G-CSF (Nicola *et al.*, 1983), to acting on multiple lineages, such as IL-3 and GM-CSF (Metcalf *et al.*, 1980; Iscove and Roitsch, 1985).

G-CSF, GM-CSF and IL-3

Studies of hematopoietic regulatory molecules in embryonic development have typically focused at stages before implantation, prior to the onset of hematopoiesis. For example, transcripts of IL-1β (Rothstein *et al.*, 1992) and IL-6 (Murray *et al.*, 1990) were detected in mouse blastocysts at 3.5 days of gestation while the mRNAs of IL-4, IL-6, SCF and *c-kit* were found during murine embryonic stem (ES) cells differentiation (Schmitt *et al.*, 1991). Although G-CSF, GM-CSF and IL-3 were well documented to be important factors in adult hematopoiesis, their expression could not be detected in 3.5 d.p.c. blastocysts or during ES cell differentiation (Rappolee *et al.*,

1988; Schmitt *et al.*, 1991). Their absence from early stages mouse embryo supports the hypothesis that G-CSF, GM-CSF and IL-3 are not critical to early hematopoiesis in fetus.

The situation in mid-late mouse embryos was revealed by the present study. The expression trend of the above mentioned hematopoietic cytokines was determined from 7.5 d.p.c. onwards, during which hematopoiesis should have occurred in the fetus. After RT-PCR, no signals for G-CSF, GM-CSF and IL-3 transcripts were obtained in embryos aged from 7.5 d.p.c. to 17.5 d.p.c.. The results are consistent with those of Kohchi's study (1994) in which no GM-CSF and IL-3 mRNAs were detected in mouse embryo from 10 to 17 d.p.c.. Although these 3 cytokines are recognized as important regulators of hematopoiesis in adult animal, they were totally absent from early blastocyst stage to late embryos. This finding further strengthens the hypothesis that G-CSF, GM-CSF and IL-3 are actually not essential in fetal hematopoiesis or development.

SCF and *c-kit*

SCF together with its receptor (*c-kit*) have been reported to have IL-3 like activities on hematopoietic CFUs (Anderson *et al.*, 1990; Martin *et al.*, 1990), and it is possible that they may have a similar function in hematopoiesis. SCF and *c-kit* were expressed through blastocyst stage to late developmental stages. Their presence implies a regulatory effect on embryonic development, properly substituting for IL-3 acting on multiple lineages. Alternative splicing gives rise to two types of SCF mRNA, SCF-l and SCF-s. Both of them encode a transmembrane domain. However, the long

form SCF (SCF-l) carries a proteolytic cleavage site which probably results into the soluble form of SCF (Flanagan *et al.*, 1991). Both types of SCF mRNA showed a gradual increase in quantity as the time of gestation grew, implying an increasing importance of activities mediated by SCF and its receptor, *c-kit*.

IL-1α, IL-1β and their receptors

As mentioned in previous section, IL-1 through IL-6 are important regulators of hematopoiesis in adult mammals. However, none of their transcripts were detected in the mouse embryos aged from mid- to late-gestation, except that of IL-1β.

IL-1α and IL-1β are the members of the IL-1 gene family. In general, they induce the same biological effects in a wide variety of immune and inflammatory responses. Their activities are mediated by the interaction with specific membrane receptors on the surface of target cells. Two types of IL-1 receptor (I and II) have been reported. Both types can bind with IL-1α or IL-1β, but with different preferences. Type II receptor (IL-1R tII) serves as a decoy receptor. By binding IL-1, the IL-1R tII can shunt the ligand away from IL-1R tI. Thus, increased expression of IL-1R tII may be a way to reduce the biological effects of IL-1. Because IL-1R tII binds preferentially to IL-1β, this type II receptor likely serves as a natural antagonist of IL-1β (Dinarello, 1994).

Using RT-PCR, amount of IL-1β transcript together with that of IL-1R tII were found to be down-regulated after 7.5 d.p.c.. IL-1R tI mRNA, on the other hand, increased as embryo grew. Previous studies indicated that IL-1R tI is found on nearly

all cell types while IL-1R tII is found on the surface of limited cell types (Dinarello, 1994). Expression of IL-1R tI more likely affects the biological responses to IL-1 since this type of receptor molecules is more able to transduce signals. Although IL-1β down-regulated, the increasing expression of IL-1R tI reflected the biological functions of IL-1 was still effective in the growing embryos.

IL-2 and receptor

IL-2 has been widely recognized as a cytokine that plays a crucial role in regulation of the immune response by exerting multiple biological activities on target cells. The best characterized activity of IL-2 is the promotion of clonal expansion of T cells (Kono *et al.*, 1990). There are 3 classes of IL-2R (α,β,γ) exist. IL-2Rα alone constitutes to the low affinity IL-2R. High affinity receptor necessarily contains all 3 component chains, IL-2Rα, β and γ (Cao *et al.*, 1993). Due to unavailability of primers specific to IL-2Rβ and γ, only the expression level of IL-2Rα was checked in this study. IL-2 transcript could not be detected but one of its receptor chain IL-2Rα was surprisingly present. Although its level of expression was low, the biological significance of their presence without the ligand is as yet unknown. This kind of situation was not rare and happened to another interleukin, IL-3.

IL-3 and receptor

IL-3, also known as a multi-colony stimulating factor is potent growth factor stimulating the development of various lineages of hematopoietic cells. The mouse IL-3 receptor (IL-3R) is composed of an IL-3Rα and a common β subunit which is shared

by IL-3, GM-CSF and IL-5 receptors. Two distinct but related genes, AIC2A and AIC2B both encode the β subunit of IL-3R (Hara and Miyajima, 1992). The expression of IL-3R(AIC2A) and IL-3R(AIC2B) were detected in embryonic tissues of the 5 developmental stages and in an increasing trend. Result for IL-3 transcript, in contrast to its receptor subunit, was negative. Similar to the case of IL-2, expression of receptor unit was observed even if the ligand was not present. The significance of such phenomenon remains unclear but is speculated that expression of receptor is for other unknown ligand that has not been discovered yet.

IL-4 and IL-6

IL-6 may play an important role *in vivo* in the expression of multiple lineages during fetal development, both by inducing non-cycling cells to become responsive to other cytokines and acting itself to support clonal proliferation and maturation of different lineages (Lee, 1992). Similarly, IL-4 also acts on various lineages of hematopoietic cells, such as lymphocytes, mast cells and basophils (Paul and Ohara, 1987). In this regard, IL-6 in combination with IL-4 stimulate colony formation of various hematopoietic progenitor cells (Rennick *et al.*, 1989). The expression of IL-4 and IL-6 were observed in blastocyst and ES cells (Murray *et al.*, 1990; Schmitt *et al.*, 1991), however, they were not detected in the mid to late stages (7.5 - 17.5 d.p.c.) of embryos (present study). The early expression of IL-4 and IL-6 implies their synergistic effects on very primitive hematopoietic cells and the effects stop at least from the onset of hematopoiesis (7.5 d.p.c.).

IL-5

In adult, IL-5 is a lineage specific cytokine which stimulates eosinophil colony formation and activates mature eosinophils. No studies have been reported for the expression patterns of IL-5 in developing embryos, therefore, its embryonic role is as yet unknown. Our results showed that the transcript of IL-5 was absent from embryo tissue of all gestational ages. It was therefore conceivable that IL-5 is not crucial in fetal hematopoiesis and that IL-5 is necessary only in times of hematopoietic crisis.

IL-10 and IL-11

The cytokine interleukin-10 (IL-10) was initially described as cytokine synthesis inhibitory factor, a product of mouse T helper 2 (Th2) clones which inhibited cytokine synthesis of Th1 clones (Fiorentino *et al.*, 1989). There were evidence indicating that IL-10 also supports the growth of hematopoietic progenitor cells, however, its potential to stimulate these cells is determined by the presence of other cytokines, like IL-3, IL-6 and G-CSF (van Vlasselaer, 1995). Due to this dependence, it is fair to assume that IL-10 acts via an indirect pathway. Similar to IL-10, IL-11 also has to act in synergy with other cytokines. For example, it supports the growth and development of primitive multipotent progenitors as well as cells committed to the megakaryocytic lineage only in the presence of SCF and IL-3 (Musashi *et al.*, 1991). Result of RT-PCR showed that neither transcript of IL-10 nor that of IL-11 was detected in any embryo tissues under investigation. These findings indicated that they were not essential or indispensable in embryonic development, probably because of their indirect and uncritical activities.

M-CSF and *c-fms*

The *c-fms* gene encodes the cell surface receptor of the growth factor M-CSF (Sherr *et al.*, 1985), and is expressed by cells of the monocyte-macrophage lineage in the hematopoietic system of the adult animal (Rettenmeier *et al.*, 1986). The interaction of M-CSF with its receptor, *c-fms*, is required for the differentiation of cells in this lineage. The present study showed that the expression level of this ligand and receptor pair in mouse embryo simultaneously increased as time of gestation increased. The coexpression of receptor and ligand suggests that M-CSF plays a role in the development and differentiation of macrophage lineage within the embryo. Using *in situ* hybridization, Regenstreif and Rossant (1989) detected the transcripts of M-CSF and *c-fms* in uterine epithelium and maternal decidua from 7.5 to 8.5 days of gestation. The fetally derived *c-fms* mRNA was observed only from 9.5 days onward in trophoblast cells while the level of M-CSF transcript in fetus was much lower than that in uterine epithelium at all later steps examined. The time course and spatial pattern of expression of these two genes suggest that the interactions between *c-fms* and its ligand M-CSF are unlikely to be important in directing events within the embryo itself until 9.5 d.p.c. when *c-fms* expression first appeared in embryos (Regenstreif and Rossant, 1989).

LIF and its transducer subunit gp130

LIF is a pleiotropic cytokine with the ability to maintain the developmental potential of pluripotent embryonic stem cells. Previous studies have revealed its expression in 3.5 d.p.c. blastocysts (Conquet and Brulet, 1990; Murray *et al.*, 1990) and uterus during the first 5 days of pregnancy (Bhatt *et al.*, 1988). Subsequent *in vivo* studies discovered that one of this protein's most important functions is to regulate implantation of the embryo by both priming the uterus and possibly regulating cell proliferation in the blastocyst (Stewart, 1994).

LIF gene transcription is discernible in the extraembryonic ectoderm of 7.5 d.p.c. embryos, and in the placenta of 9.5, 10.5 and 12.5 days of gestation (Conquet and Brulet, 1990). In this study, LIF mRNA was detected in all embryos aged from mid- to late gestation (7.5 to 17.5 d.p.c.), though with a down-regulating trend. Its continued presence throughout gestation further implicates this molecule in embryogenesis. Moreover, differential expression of LIF transcript is further modulated by other cytokines such as IL-1α, IL-1β, TGF-β and bFGF (Kurzrock *et al.*, 1991), a phenomenon that may be responsible for fine-tuning control of LIF actions in different physiological situations.

LIF receptor (LIFR) belongs to hematopoietin receptor family which can be generated as secreted extracellular proteins via alternative splicing (Mosley *et al.*, 1989). The long form LIFR cDNA includes a stop codon ahead of the transmembrane and cytosolic domains and thus encoding soluble form of LIF receptor molecules. Secreted receptors can act as natural antagonists of cytokine action by directly

competing for ligand with transmembrane receptor and/or by promoting the systemic clearance of secreted cytokines (Goodwin *et al.*, 1990). In this manner, soluble form of LIFR could be an important determinant of embryonic differentiation. Long form and short form of LIFR mRNA appeared to express at fairly constant level in the embryonic tissue along the 5 developmental stages, however, the level of membrane bound form (LIFR-s) was generally lower than that of its secreted counterparts.

The physiological effect initiated by LIF is, therefore, not only adjusted by its production but also by the presence of natural antagonist (soluble receptor) and specific regulators such as IL-1, TGF-β and bFGF.

gp130 is a ubiquitously expressed signal-transducing receptor component shared by LIF, IL-6, IL-11, CNTF, OSM and cardiotrophin-1 (Kishimoto *et al.*, 1996). The discovery of this shared signal transducer helps to explain how these different cytokines mediate overlapping biological functions. The present study revealed that the levels of gp130 transcript were fairly constant through 7.5 to 17.5 d.p.c., both in embryo and placenta. Yoshida and his co-workers (1996) provided evidence for the fact that gp130 is essential in embryonic development. They created mice deficient in gp130 by targeted disruption of the gene and found that embryos homozygous for the mutation progressively died between 12.5 d.p.c. and term. The knockout mice experiment reflects the importance of this signal transducer in development.

2.5.4.2 Other cytokines

TGF-β

TGF-β is a family of polypeptides that regulate cell growth and differentiation. It is now apparent that at least five genetically distinct forms of TGF-β exit. In this study, it was the transcript of TGF-β1, which is the most predominant isoform, being investigated. Its mRNA was detected in mouse embryos from day 7.5 onwards with a gradual increasing quantity. This finding is consistent with the results of other previous studies, in which TGF-β1 mRNA or protein was also observed from mid- to late gestation (Heine *et al.*, 1987; Lehnert and Akhurst, 1988). Expression of TGF-β1 in defined temporal pattern during mouse development reflects its distinct role in fetal development. The inactivation of the mouse TGF-β1 gene has been shown to result in a wasting syndrome with diffuse inflammatory infiltrates in homozygous animals which died before or right after birth (Kulkarni *et al.*, 1993). Lack of this cytokine is therefore detrimental to mouse.

TNF-α and TNF-β

These two cytokines are multifunctional and implicated in diverse processes, including acute inflammation, angiogenesis, blood cell differentiation, cell proliferation and cell killing. Since TNF-α is defined to be a molecule orchestrating inflammation in adult, it was then hypothesized that the major roles of TNF-α in embryonic development are to induce a state of so-called 'ontogenic inflammation' (Yamasu *et al.*, 1989), which is an essential pre-requisite for normal embryonic development. Kohchi and co-workers (1994) demonstrated that both TNF-α and TNF-β were expressed

constitutively in almost all organs of fetus as well as the placenta from 10th day of gestation and onwards. He then postulated that both TNF-α and TNF-β are molecules involved in 'ontogenic inflammation'. The present study also detected the presence of TNF-α mRNA from 7.5 to 17.5 d.p.c.. The level of TNF-α down-regulated at 9.5 d.p.c. but increased afterwards. Evidence provided that either an excess or too little amount of TNF-α might be fatal, therefore its expression has to be fine tuned in a time-dependent pattern for normal development. In contrast to Kohchi's study, transcript of TNF-β could not be detected here. It is therefore speculated that TNF-β may not be critical in 'ontogenic inflammation'. Since there is no other report on TNF-β in connection with embryonic development, the role of TNF-β in this context remains controversial.

CNTF

CNTF is a cytokine that promotes the survival of ciliary, sensory and sympathetic and motor neurons *in vitro* (Sendtner *et al.*, 1994). Stewart and co-workers (1994) have been unable to detect CNTF transcripts in the mouse uterus or pre-implantation embryo. On the other hand, its mRNA was detected in mouse embryos of mid- to late gestational ages (present study). CNTF knockout mice were derived and were overtly normal during the first postnatal weeks. But with increasing age, spinal motor neurons exhibited progressive degeneration which was functionally reflected by a significant reduction in muscle strength (Masu *et al.*, 1993). This result has demonstrated that expression of CNTF gene is not necessary for the maintenance of motor neuron function during embryonic development, but it has an essential role in regulating motor neurons in the postnatal period. It seems that the role of CNTF in

embryogenesis remains elusive, however, the presence of its transcript in embryonic tissue and its ability to maintain the pluripotentiality of ES cells (Conover *et al.*, 1993) suggest a functional role of CNTF in mammalian development.

IFN-γ

IFN-γ has potent immunoregulatory effects on a variety of cells including activation of macrophages, increase in natural killer cell activity, up-regulation of antibody secretion, etc. It is clear that IFN-γ plays important role in the immune system but what role does it play in embryonic development? Rothstein *et al.* (1992) found the expression of IFN-γ in blastocyst of mouse embryo. It was shown in this experiment, however, that the expression of this cytokine could not be detected in embryos aged from 7.5 to 17.5 d.p.c.. This result is identical to the finding by Kohchi and his co-workers (1994), in which IFN-γ transcript could not be found in mouse embryos of mid-late gestation (10 to 17 d.p.c.). Therefore, the expression of IFN-γ was not constitutive throughout development, but only occurs transiently during early embryogenesis and stops at least from 7.5 d.p.c. onwards. The teleological meaning of this transient expression of IFN-γ awaits further experimentation.

2.5.5 Expression Pattern in placenta: maternal and fetal communication

Cytokines have key roles in fetal-maternal relationships during pregnancy. Cytokine expressed mainly in maternal tissues would have the task of preparing a good environment for the embryo, whereas cytokines expressed in embryo could function for the embryo's own development. The supply of maternal cytokines would become less substantial and influential as the embryo could generate such molecules by itself. As observed from the expression profile of cytokines in placenta, most of them were gradually down-regulated as embryo developed and grew. Exception was also obtained, in which SCF-l, SCF-s and *c-fms* transcripts showed an increasing amount in placenta as gestation period prolonged, similar to the trend observed in embryos.

Chapter 3

Molecular analysis of mouse Oncostatin M

3.1 Introduction

Human OSM gene had been cloned and sequenced early in 1989. However, the mouse OSM gene is not studied up to date. According to gene structure, primary amino acid sequence, biological functions and receptor binding, OSM is grouped into a cytokine subfamily named as neuropoietic family. The group also includes LIF, CNTF, IL-6 and IL-11. Among the members, OSM is more closely related to LIF. In view of LIF, the human and mouse clone have a high homology of nearly 90%. A similar high homology is predicted for OSM gene. Therefore, it is reasonable to use human OSM exon fragments to fish out the mouse clone.

The aim of this part of the project is to perform molecular analysis of the murine OSM gene. The result may facilitate further studies on this cytokine using mouse model.

The analysis procedures for this part of the thesis was summarized in **Figure 3.1**. As the gene sequence of human OSM was documented, two pairs of primer were specially designed to clone two different exon fragments by using PCR method. Their priming sites were schematically represented in **Figure 3.2**. The first pair of the primers flanked exon 2 with a product size of 121 bp while the other pair flanked

exon 3 coding region and gave a fragment of 591 bp in length. The PCR products were then subcloned into vector for manipulation. The identity of the subcloned fragments were directly verified by sequencing. After labeling the purified hOSM exon 2 and exon 3 fragments, Southern hybridization against mouse genomic DNAs was performed separately. Human genomic DNA was also Southern hybridized in parallel as a control experiment.

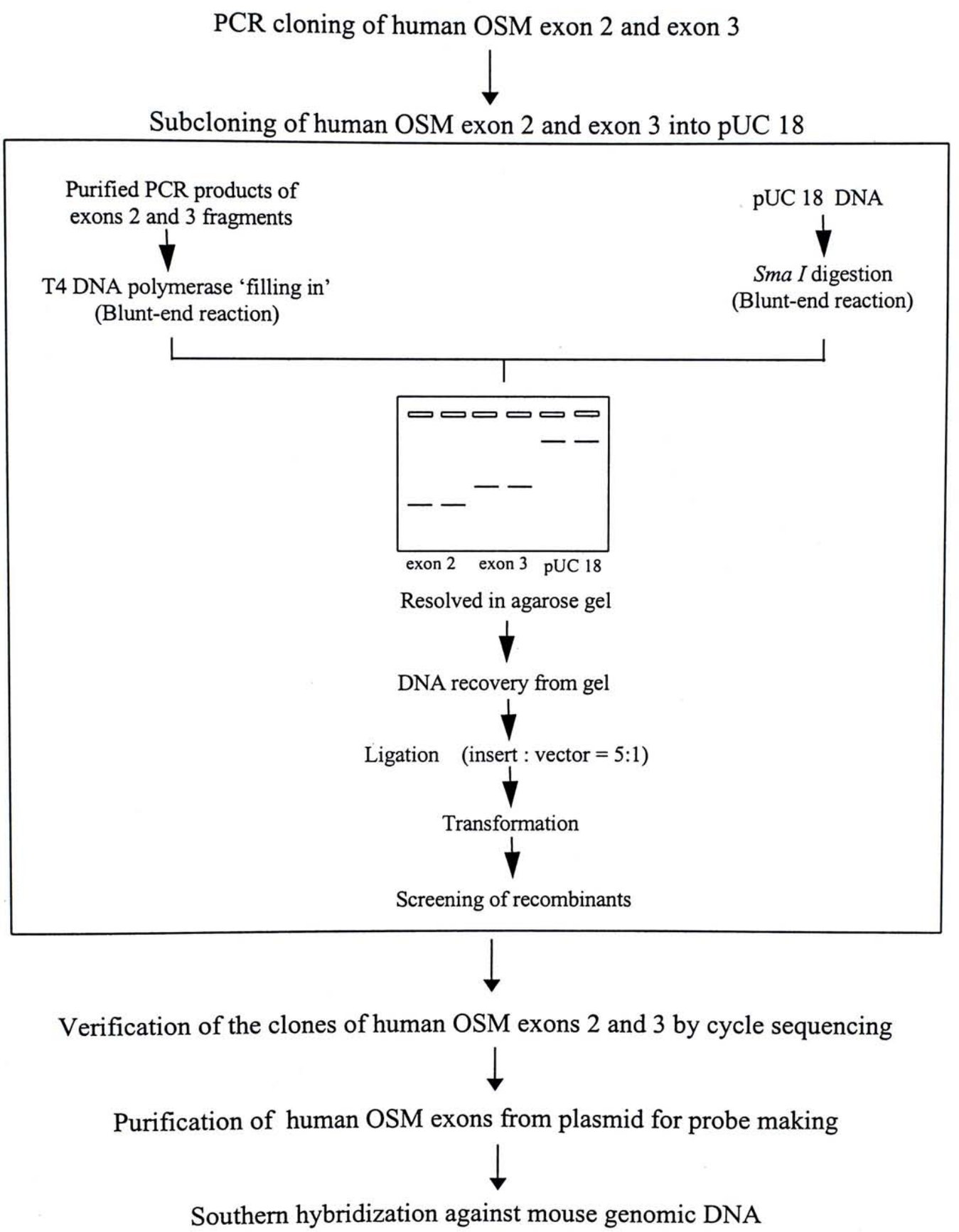


Figure 3.1 Flowchart of the analysis procedures for mouse OSM gene.

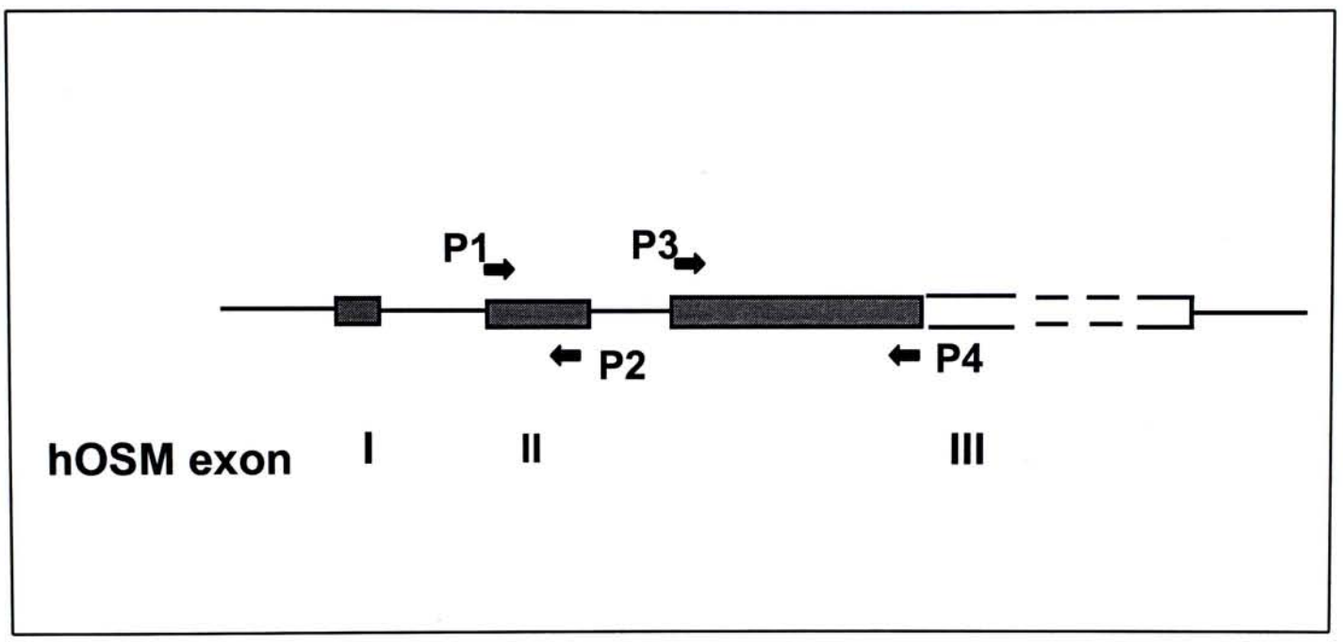


Figure 3.2 The priming sites of the two pairs of specially designed primers for human OSM exon 2 and exon 3 fragments. P1 and P2 flanked a fragment 121 bp in length while P3 and P4 amplified a 591 bp-fragment. The unfilled portion of exon III represents the 3' non-coding region.

3.2 Materials

3.2.1 Chemicals and Reagents

Ampicillin	Sigma (No. A-9518)
Ammonium acetate (NH_4OAc)	Sigma (No. A-8750)
5-Bromo-4-chloro-3-indolyl-β-D galactoside (X-gal)	Sigma (No. B-4252)
Cetyltrimethylammonium bromide (CTAB)	Sigma (No. H-5882)
Chloroform	Ajax chemicals (No. 152)
Dodecyltrimethylammonium bromide (DTAB)	Sigma (No. D-8638)
Formamide	Sigma (No. F-7503)
Isoamylalcohol	Ajax chemicals (No. 64)
Isopropyl-β-D-thiogalactoside (IPTG)	Sigma (No. I-6758)
MOPS	Sigma (No. M-8899)
N,N-Dimethylformamide	Sigma (No. D-8654)
Nick column, Sephadex G50	Pharmacia(No.17-0855-02)
Phenol	Sigma (No. P-3653)
Polyethylene glycol MW. 8000 (PEG 8000)	Sigma (No. P-2139)
Redivue [α -^{32}P] dCTP, 3000 Ci/mmol	Amersham (No. AA 0005)
Rubidium chloride (RbCl)	Sigma (No. R-2252)
Triton X-100	Fluka (No. 93426)
Tryptone	Oxoid (code L42)
Yeast extract	Oxoid (code L21)

Remark: The water used throughout the experiments was of ultrapure grade.

3.2.2 Enzymes

Lysozyme	Sigma (No. L-6876)
Proteinase K	Sigma (No. P-4914)

T4 DNA ligase (8,500 Weiss units/ml, Pharmacia)

T4 DNA polymerase (10 U/ µl, Pharmacia)

Restriction enzymes:	*Bam* H I	50 U/ µl, Amersham
	*Eco*R I	12 U/ µl, Promega

*Eco*R I	60 U/ µl, Promega
Pst I	60 U/ µl, Amersham
Sma I	12 U/ µl, Promega
Hind III	80 U/ µl, Promega
RNase ONE	10 U/ µl, Promega

3.2.3 Buffers

Buffer H for *Eco*R I, 10x	Promega
Buffer H for *Pst* I, 10x	Amersham
Buffer J for *Sma* I, 10x	Promega
Multi-core buffer, 10x	Promega
ONE-PHOR-ALL buffer	Pharmacia

TE buffer : 10mM Tris-HCl (pH 7.5) and 1mM EDTA (pH 8.0)

3.2.4 Solutions

Ampicillin stock solution

50 mg/ml stock solution was prepared by dissolving 1g of the sodium salt of ampicillin in 20 ml H_2O. The solution was sterilized by filtration and stored at -20°C.

CTAB solution

5% CTAB, 0.4M NaCl

DTAB solution

8% DTAB, 1.5M NaCl, 100mM Tris-HCl pH 8.6, 50mM EDTA

IPTG solution

A 0.5M stock solution was prepared by dissolving 1.4 g of IPTG in 10 ml distilled water. The solution was sterilized by filtration through a 0.22 μm membrane and stored at -20°C.

PEG solution

30% PEG 8000, 1.8M NaCl

Phenol equilibrated with Tris-buffer

Phenol was equilibrated to a pH > 7.8 before use. Equal volume of Tris-HCl (pH 8.0) was added to liquified phenol and mixed well. Aqueous phase was removed after equilibration.

RF 1 solution

RF 1 was composed of 0.1M RbCl, 50mM $MnCl_2 \cdot 4H_2O$, 30mM potassium acetate, 10mM $CaCl_2$ and 15% (w/v) glycerol. The final pH of the solution was adjusted to 5.8 with 0.2M acetic acid. Sterilization was done by filtration.

RF 2 solution

RF 2 contained 10mM MOPS, 10mM RbCl, 75mM $CaCl_2 \cdot 2H_2O$ and 15% (w/v) glycerol. The final pH was adjusted to pH 6.8 with NaOH (as necessary) and the solution was sterilized by filtration.

STET.1 solution

STET.1 solution was made up of 8% sucrose, 50mM EDTA, 10mM Tris-HCl (pH 8) and 0.1% Triton X-100.

X-gal stock solution

250mg/ml stock solution was prepared by dissolving 1g of X-gal in 4 ml N,N-dimethylformamide (DMF). The stock was stored at -20°C.

3.2.5 Culture media

Luria-Bertani (LB) medium

LB medium contained 10g tryptone, 5g yeast extract and 10g NaCl per liter. It was sterilized by autoclaving for 20 min at 151 lb/sq. in. on liquid cycle.

LB agar

LB medium added with 15g agar per liter.

SOB medium

One liter of SOB contained 20g tryptone, 5g yeast extract, 0.5g NaCl and 2.5mM KCl. The pH was adjusted to 7.5 with NaOH and sterilized by autoclaving. 5ml of a sterile solution of 2M $MgCl_2$ was added afterwards.

3.2.6 Competent cell

DH5α was used for making competent cell. Its genotype was F^- ϕ80d *lac*ZΔM15 Δ(*lac*ZYA-*arg*F) U169 *deo*R *rec*A *end*A1 *hsd*R17(r_K^-,m_K^+) *sup*E44λ^- *thi*-1 *gyr*A96 *rel*A1.

3.2.7 DNA materials

1 kb ladder DNA marker (GIBCO/BRL No. 15615-016)

λ DNA/*Hin*d III marker

Human genomic DNA (Promega No. G 3041)

Mouse genomic DNAs (Promega No. G 3091)

Mouse genomic DNAs of BALB/c and C57BL

pUC 18

3.2.8 Primers

Table 3.1 Sequence of primer specific for human OSM exons 2 and 3.

mRNA		sequences (5' to 3')	size of fragment (bp)
hOSM exon 3	upper primer	CAAGGCCTGGATGTTCCTAAAC	591
	lower primer	CAAGGGGTGCTCTCGAGGCT	
hOSM exon 2	upper primer	GTCTGGTCCTTGCACTCCTGTT	121
	lower primer	GTGTCCTGCATGAGATCTGTCT	

3.3 Methods

3.3.1 Primers and internal probes

Oligonucleotide primers were custom synthesized by Integrated DNA Technologies, Inc. The oligonucleotides were purified by gel filtration column and obtained as lyophilized powder.

Upon arrival, powder of primer was first dissolved in 500 µl $TE_{0.1}$ buffer and the concentration was determined by OD measurement at 260nm according to the following formula:

Concentration of oligonucleotide (pmol/ µl) = OD_{260} / ε x dilution factor x 1000

where ε corresponds to the summation of extinction coefficient of the bases in an oligomer.

(A = 11.5, T = 8.8, G = 11.7, C = 7.3)

A working solution of 25 pmol/ µl of each primer was prepared.

3.3.2 Cloning of human Oncostatin M exon 2 and exon 3 by PCR

Primer pairs specific for human OSM exon 2 and exon 3 were used to clone the exon fragments from the human genome by PCR method. A volume of 20 µl

containing ~1μg of human genomic DNA was first boiled for 10 min and then quickly chilled on ice. The PCR was initiated by adding the boiled DNA template to 30 μl reaction mixture containing 5 μl of 10x PCR buffer [20mM $(NH_4)_2SO_4$, 75mM Tris-HCl, pH 9.0 and 0.01% (w/v) Tween], 3 μl of 25 mM $MgCl_2$, 1 μl of 10mM dNTP mix, 2 μl of each corresponding primers (25 pmol/ μl), 0.5 μl of 0.5 U/ μl of Thermoprime^{plus} DNA polymerase (Advanced Biotechnologies) and 16.5 μl sterile double distilled water. The reaction mixture was then overlaid with mineral oil and subjected to amplification using the Temp'Tronic thermocycler (Thermolyne Co.). The temperature profile was 94°C for 1 min; 56°C for 1 min; 72°C for 1 min, and was repeated for 30 or 50 times.

20 μl of the PCR products was analyzed by gel electrophoresis in 1.5% Synergel-0.7% agarose using 1x TAE buffer containing (0.5 μg/ml) EtBr. After electrophoresis, the EtBr-stained DNA bands were visualized and photographed under UV illumination.

3.3.3 Subcloning of human OSM exons 2 and 3 into pUC 18

a. Preparation of human OSM exons and plasmid

i) Purification of PCR products

Putative human OSM exon 2 and exon 3 cloned by PCR method (10 reactions for either primer pair were first pooled into one tube) were purified by

phenol:chloroform:isoamyl alcohol (25:24:1) extraction. The DNA fragments were then precipitated using 0.5 volume of 7.5M NH_4OAc and 2.5 volume of absolute ethanol. The pellets of exon 2 and exon 3 fragments were resuspended in 50 µl sterile water separately.

ii) T4 DNA polymerase 'blunt-end' reaction for PCR products

DNA fragments resulted from PCR were blunt-ended by using T4 DNA polymerase. The reaction was carried out in a mixture containing 50 µl of purified PCR product, 20 µl of 5x T4 DNA polymerase buffer (0.165M Tris-acetate pH8.0, 0.33M potassium acetate, 0.05M magnesium acetate, 2.5mM DTT and 0.5 mg/ml bovine serum albumin) and 26 µl sterile water. The mixture was incubated at 37°C and 20 U of T4 DNA polymerase was added after 5 min of incubation. In not more than 30 seconds, 2 µl of 10mM dNTPs was also added to the reaction mixture which was then incubated for another 30 min at 37°C.

iii) Sma I digestion of pUC 18

The vector, plasmid pUC 18, was digested by *Sma* I enzyme to create a linear fragment with blunt ends. 5 µg of pUC 18 was digested by 8 U of *Sma* I in 1x Buffer J (Promega) at 25°C for an hour.

b. Ligation

Ligation was carried out in an insert to vector ratio of 5:1. The amount of insert and vector was estimated by comparing the intensity of band resolved on gel.

Linearized pUC 18 (2,686 bp) and purified human OSM exon 3 DNA fragments were electrophoresed on 1% (w/v) agarose while smaller human OSM exon 2 DNA fragments were resolved on 1.5% Synergel-0.7% agarose. The DNA molecules were recovered from gel by squeezing the gel slices with centrifugal force across a 3MM Whatman filter paper. The DNA suspension was then purified and extracted with chloroform and finally precipitated with ethanol and sodium acetate.

Ligation reactions were carried out for exon 3 or exon 2 and *Sma* I cut pUC 18 and *Sma* I cut pUC 18 alone. A 20-µl reaction mixture containing appropriate amount of insert and vector or vector alone, 1x ONE-PHOR-ALL buffer (100mM Tris-acetate, 100mM magnesium acetate, 500mM potassium acetate), 17 U of T4 DNA ligase (Pharmacia Inc.) and 1mM ATP was incubated at 16°C overnight. After incubation, the ligation products were purified by chloroform extraction and ethanol precipitation. The pellets were then resuspended in 10 µl sterile water.

c. Preparation of competent cell

E. coli strain DH 5α were used for preparing competent cells. Single colony was picked from a freshly streaked LB agar plate and dispersed in 1 ml SOB medium by vortexing. The suspension was inoculated into an Erlenmeyer flask containing 30 ml SOB medium which was then incubated at 37°C with moderate agitation until the cell density reached 4 x 10^7 viable cells/ ml (OD_{550} = 0.4). The culture was chilled on ice for 15 min before centrifugation at 1000 g for 15 min at 4°C. The pellet was resuspended in RF 1 solution (1/3 x collected volume) and incubated on ice for 30 min.

Centrifugation step was repeated and this time the resulting pellet was resuspended in RF 2 solution (1/12.5 of the original volume) which was later incubated on ice for 15 min. Aliquots of the competent cells were then distributed into pre-chilled screw cap tubes and immediately flash freezed in liquid nitrogen. The tubes were stored at -70°C.

d. Transformation

150 µl of competent cells was added to each 10 µl purified ligation product and mixed well. The tubes were incubated on ice for 60 min, followed by heat shock in a 42°C water bath for 2 min and immediately another 1-min incubation on ice. 800 µl of LB medium was added and incubated at 37°C with moderate agitation for 60 min. Different volumes (50 µl, 100 µl, 200 µl and rest) of the culture were then spreaded on LB agar plates supplemented with ampicillin (100 µg/ml), X-gal and IPTG. The plates were incubated at 37°C overnight.

e. Screening of recombinants by PCR

Thirty white colonies from each batch (exon 2 or exon 3 insert) appeared on LB^{AMP}-X-gal-IPTG plates were picked by sterile tooth-picks and resuspended in 50 µl water. Bacterial cells were heat lyzed by boiling for 10 min and quickly chilled on ice/NaCl. 20 µl of the solution was subjected to PCR with conditions as described in cloning section (*Section 3.3.2*). The PCR products were analyzed by gel electrophoresis.

f. Screening of recombinants by restriction enzyme digestion

i) Preparation of plasmids

Several recombinant colonies (white in color and gave appropriate PCR products in previous section) were separately cultured in 30 ml LB medium at 37°C overnight for plasmid DNA extraction. The bacterial cells were transferred to Corex tubes and centrifuged at 5,000 rpm for 10 min in a JA-20 rotor (Beckman). The pellets were resuspended in STET.1 solution and added with 4 mg of lysozyme (Sigma). After mixing, the solution was boiled for 60 seconds and immediately centrifuged at 15,000g at 4°C for an hour. The supernatant was then transferred to a new microcentrifuge tube and its volume was adjusted to 900 µl with TE buffer. The solution was sequentially treated with 10 U RNase ONE (37°C, 1 hour) and 1 mg of proteinase K (50°C, 30 min). For 1 ml of solution, 400 µl PEG solution (30% PEG 8000, 1.8M NaCl) was added and the mixture was incubated on ice for 30 min. Pellets were recovered by centrifugation at 15,000g at 4°C for 30 min and resuspended in 450 µl water. 50 µl of CTAB solution (5% CTAB in 0.4 M NaCl) was added to pellet DNA molecules by centrifugation for 15 min at room temperature. The CTAB-pellets were washed with 1.2 M NaCl solution which were then re-precipitated out by centrifugation. The pellets at this stage were resuspended in 200 µl water and finally precipitated with 0.5x volume of 7.5M NH_4OAc and 2.5x volume of absolute ethanol. The resulting pellets were resuspended in 100 µl $TE_{0.1}$ buffer and stored at -20°C. The concentrations of plasmid were determined by spectrophotometric method in which 1 unit of OD_{260} corresponds to 50 µg/ml ds DNA.

ii) Double restriction enzymes digestion of pUC 18

To test if the clones of vector carried the insert of interest, purified plasmids from above were simultaneously digested with *Eco*R I and *Bam* H I. The cutting sites of pUC 18 by these two restriction enzymes were shown in **Figure 3.3**. Approximately 10 U of each enzyme was used to digest 1 μg of plasmid DNA in 1x Multi-Core buffer (25mM Tris-acetate pH7.8, 100mM potassium acetate, 10mM magnesium acetate and 1mM DTT) at 37°C for an hour. The products of digestion were then separated on agarose gel. If pUC 18 contained the subcloned human OSM exon 2 or exon 3, there would be a band of ~140 bp or ~610 bp in length respectively, in addition to the band of linearized pUC 18 (~ 2.7kb).

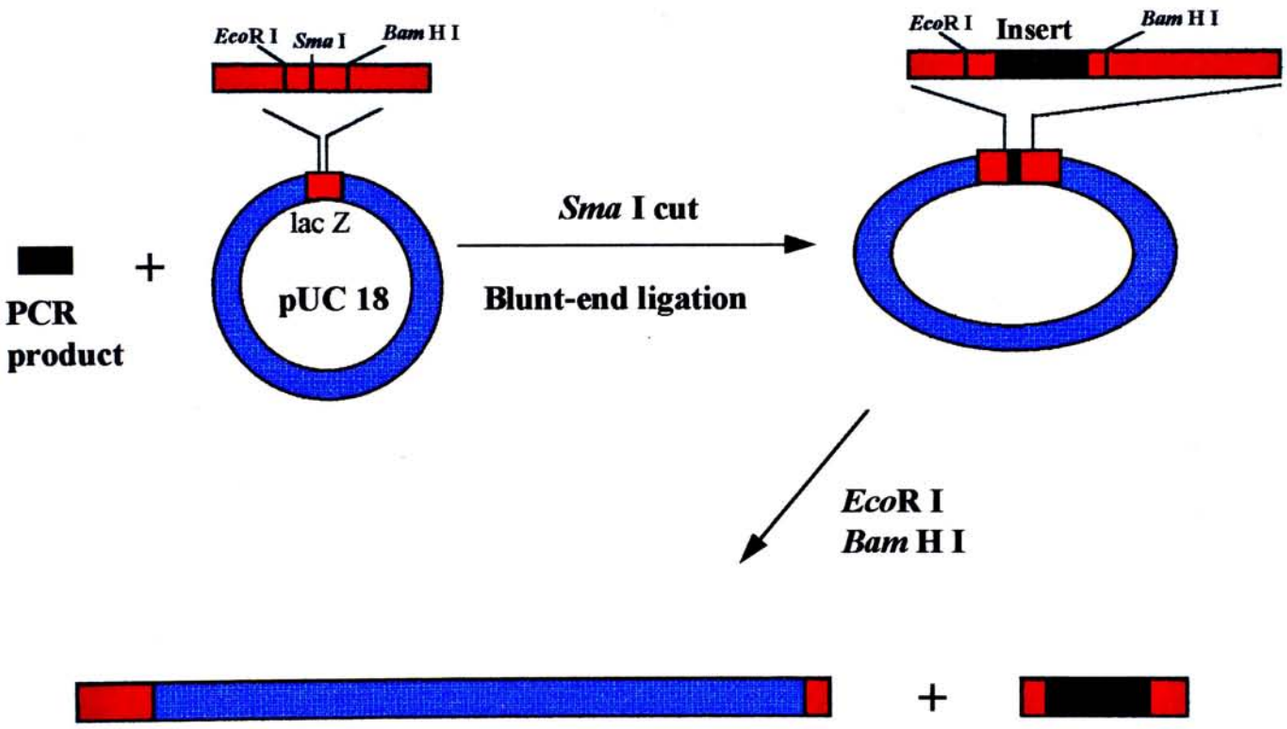


Figure 3.3 Subcloning of exon fragment into vector and screening of recombinant by restriction enzyme digestion. Resultant cut fragments were analyzed by electrophoresis on agarose gel to check their respective sizes.

3.3.4 Verification of the clones of human OSM exons 2 and 3 by cycle sequencing

The identity of the putative human OSM exons 2 and 3 subcloned into pUC 18 was verified by sequencing the two DNA fragments. Cycle sequencing using dideoxy chain terminators was adopted in this experiment. The sequencing primer used was M13/pUC Forward 23-Base Sequencing Primer (Gibco/BRL). The sequencing process was done using dsDNA cycle sequencing kit purchased from Gibco/BRL. The reaction was initiated by mixing the following: 24 μl water, 4.5 μl 10x buffer (300mM Tris-HCl pH9.0, 50mM $MgCl_2$, 300mM KCl, 0.5% (w/v) W-1), 1 μl purified pUC 18 with putative exon 2 or exon 3 insert, 6 μl 4mM sequencing primer and 0.5 μl of 1.25 U/ μl Taq DNA polymerase.

8 μl of the reaction mix was pipetted to 4 separate microcentrifuge tubes which contained 2 μl of one of the four types of termination mix (2mM either of ddATP, ddCTP, ddGTP or ddTTP and 100 μM of each dATP, dCTP, deaza-dGTP and dTTP). The tubes were then overlaid with mineral oil and subjected to a temperature profile of 95°C for 36 sec, 50°C for 36 sec and 72°C for 84 sec. The profile was repeated for 25 times and followed by a temperature extension at 72°C for 10 min.

10 μl sequencing products of each sample was resolved by 6% urea-polyacrylamide gel operated in a Pharmacia ALF DNA sequencer. The sequencing products were denatured at 95°C for 3 min and then chilled on ice before gel separation. The sequencing gel was run at 40°C of 34 W for 6 hours and the running buffer used was 0.6x TBE. The laser power was set to 3 mW.

The raw DNA sequences of putative human exon 2 and exon 3 were edited by Pharmacia ALF manager and the edited DNA sequences were compared against the GenBank nucleotide and protein databases via the NCBI BLAST Electronic-mail service.

3.3.5 Purification of human OSM exons from plasmid for making probe

Human OSM exons subcloned into pUC 18 were first excised and purified from the vector before probe making. pUC 18 carrying exon 2 and exon 3 were separately digested with both *Eco*R I and *Bam* H I in 1x Multi-Core buffer, as described in *Section 3.3.3 f(ii)*. The products were resolved in 2% (w/v) agarose gel and the bands corresponding to exons 2 and 3 were cut out. The DNA fragments were then extracted and purified following the procedures depicted in *Section 3.3.3 a (i)*. The quantity of recovered DNA was estimated by comparing the intensity of EtBr-stained bands with known amounts of the corresponding exon fragment.

3.3.6 Southern blotting

a. Probe making and labeling

The probes of human OSM exon 2 and exon 3 were made and labeled by PCR method. The templates used were purified exon fragments excised from plasmid pUC

18. ^{32}P-dCTP was incorporated into PCR using the same pair of specific primer used in PCR cloning (*Section 3.2.8*).

The composition of a labeling reaction in a final volume of 50 µl was 0.2 ng of template, 15mM Tris-HCl (pH 8.3), 1x buffer, 0.25 U of Thermoprime^{plus} DNA polymerase, 1 µM of each primer, 200 µM of each dATP, dCTP and dGTP, 3.2 µM dCTP and 0.8 µM ^{32}P-dCTP. The reaction was overlaid with mineral oil and was subjected to 30 cycles of amplification (94°C, 1 min; 56°C, 1 min; 72°C, 1min) following denaturation at 94°C for 2 min.

After amplification, PCR products were separated from primers and unincorporated nucleotides through NICK column, Sephadex G50 (Pharmacia) following the procedures of its instruction manual. The percentage of incorporation of ^{32}P-dCTP therefore the yield of PCR was estimated by scintillation counting of samples before and after NICK column separation.

b. Preparation of mouse genomic DNAs

The protocol for preparing genomic DNA was modified from Xu *et al.*, 1990. The spleens of mouse strains BALB/c and C57BL were dissected out and washed with PBS. Single cell suspension was made by passing through sterile stainless steel mesh. Cell pellets were obtained by centrifugation and resuspended in 0.5 ml PBS. The cell suspension was then added dropwise to a Petri dish containing 4 ml guanidinium thiocyanate (GT) solution (4M GT in 0.1M Tris-HCl pH 7.5) and 0.1M NaOAc with

gentle shaking. 2 ml of the cell lysate was then carefully layered under 10 ml of absolute ethanol (EtOH) in a 15-ml centrifuge tube using a large bore siliconized Pasteur pipette wetted with EtOH for transfer. The DNA was then recovered by rolling the tube horizontally until white threads of condensed nucleic acids were observed. The tube was further gently inverted several times to thoroughly mix the EtOH and aqueous phases and to pack the threads tightly. The DNA pellets were sequentially transferred to tubes of 70 % and 100% EtOH and finally dried in air. The DNA was then dissolved in 2 ml TE buffer with 2 % SDS and treated with 100 U RNase ONE (37°C, 2 hours) and subsequently with 100 µg proteinase K (37°C, overnight). The DNA was recovered as before by adding 10 ml EtOH. The pellet was finally resuspended in 2 ml $TE_{0.1}$ buffer. Incubation at 65°C was generally needed to facilitate dissolution of pellets.

Concentration and quality of genomic DNA were determined by spectrophotometric measurement and agarose gel electrophoresis.

c. DNA transfer

i) Digestion of genomic DNA with restriction endonucleases

Large and complex genomic DNAs have to be digested by restriction enzyme into smaller fragments before blotted on membrane. 10 - 15 µg of each type of genomic DNA was digested with restriction endonucleases, *Eco*R I, *Hin*d III and *Pst* I. Digestion was carried out with 10 U of each restriction enzyme per µg DNA in 1x

corresponding buffer and incubated at 37°C for at least 6 hours with occasional gentle mixing. The digestion mixture was then extracted with chloroform and concentrated into 30 µl $TE_{0.1}$ buffer with ethanol precipitation.

ii) Gel electrophoresis and DNA blotting

The 30 µl digested DNA and molecular size markers were loaded into performed well in 0.8 % (w/v) agarose gel and electrophoresed at 1 V/ cm of gel length in 1x TAE buffer containing 0.5 µg/ml EtBr. The subsequent procedures of DNA transfer to nylon membrane was depicted in *Section 2.3.6 a.*

d. Hybridization

The baked nylon membrane with DNA was placed into a roller bottle. 50 ml of hybridization buffer (*section 2.2.4*) was added and prehybridization was performed for at least 4 hours at 68°C for human DNA blot and at 60°C for mouse DNA blots with constant rotation in a hybridization oven (Robbins Scientific Co.).

After prehybridization, the solution was replaced by ~ 10 ml of hybridization buffer containing 5 - 10 ng/ml radiolabeled human OSM exon 2 or exon 3 fragments which were freshly heat denatured. Hybridization was carried out for 18 hours at 68°C and 24 hours at 60°C for human DNA blot and for mouse blots respectively.

Washing steps of nylon membranes after hybridization were different for human DNA blot and mouse blots. They were listed as follows:

Human OSM exon 3 vs Human genomic DNA blot		**Human OSM exon 3 vs Mouse genomic DNA blot**	
		5XSSC; 0.1% SDS	2 x 15min at 60°C
2XSSC; 0.1% SDS	2 x 15 min at RT	2XSSC; 0.1% SDS	2 x 15 min at 60°C
0.1X SSC; 0.1% SDS	2 x 15 min at 68°C	1X SSC; 0.1% SDS	2 x 15 min at 60°C

Human OSM exon 2 vs Mouse genomic DNA blots			
2X	1X	0.5X	0.1X
5X[a] , 2x 15min at 60°C	5X , 2x15min at 60°C	5X , 2x15min at 60°C	5X , 2x15min at 60°C
2X[b], 2x15min at 60°C	2X, 2x15min at 60°C	2X, 2x15min at 60°C	2X, 2x15min at 60°C
	1X[c], 1x15min at 60°C	1X, 1x15min at 60°C	1X, 1x15min at 60°C
		0.5X[d], 1x15min at 60°C	0.5X, 1x15min at 60°C
			0.1X[e], 1x15min at 60°C

[a] 5X SSC; 0.1% SDS [b] 2X SSC; 0.1% SDS [c] 1X SSC; 0.1% SDS [d] 0.5X SSC; 0.1% SDS
[e] 0.1X SSC; 0.1% SDS

The background was checked by Mini-monitor (Mini-instruments Ltd., type 5.10). If the count was over 10 counts per second, the membranes were washed for longer time until the background dropped below 5 counts per second.

The washed membranes were blotted briefly between 3MM paper and secured within Saran wrap. The membranes were then fixed onto a X-omatic cassette with an intensifying screen (Kodak) and exposed to X-ray film (Kodak, X-omatic AR) at -70°C for 24 hours.

The X-ray films were developed manually following the procedures of 5 min in GBX X-ray developer and replenisher (Kodak), 1 min in running water, 5 min in fixer and replenisher (Kodak) and finally 15 min in running water.

The molecular size of hybridization signal on exposed X-ray film was estimated by measuring the distance between the sample-loading well and the signal. A standard curve was drawn by the molecular size of marker standards against the migration distance in a semi-log scale format. Molecular size of the signal was determined by interpolating its migration distance.

3.4 Results

3.4.1 Cloning of human OSM exon 2 and exon 3 by PCR

Human OSM exons 2 and 3 fragments were cloned by PCR method using 2 separate pairs of primers. The PCR products were analyzed on Synergel-agarose gel (**Figure 3.4**). The sizes of the amplified exon 2 and exon 3 fragments were 121 bp and 591 bp respectively.

3.4.2 Subcloning of human OSM exons 2 and 3 into pUC 18

Purified PCR products of exon 2 and exon 3 were end-filled by T4 DNA polymerase to create blunt ends. The fragments were separately ligated to *Sma* I cut pUC 18. A control experiment which contained *Sma* I cut pUC 18 only was done in parallel. Bacterial colonies appeared on LB^{AMP}-X-gal-IPTG plates were either white (recombinant) or blue (non-recombinant) in color. For the control experiment, all bacterial colonies were blue, indicating normal functioning of the *lacZ* gene. The transformation efficiencies for both exon 2-pUC 18 and exon 3-pUC 18 were over 90% as observed from the ratio of white to blue colonies.

a. Screening of recombinants by PCR

Identity of the recombinants were first checked by PCR. 30 white bacterial colonies from each batch (exon 2 or exon 3 insert) appeared on LB^{AMP}-X-gal-IPTG

plates were picked out and subjected to PCR and the products were electrophoresed on agarose gel (**Figure 3.5**).

The product size should be either 591 bp or 121 bp if the recombinant bacteria truly carried the OSM exon fragments. For exon 3, 20 out of 30 white colonies gave out a product fragment around 591 while for exon 2, the portion of true recombinant was rather low, just 7 out of 30.

b. Screening of recombinants by restriction enzymes digestion

Only those bacterial clones which carried appropriate exon fragments were further tested by restriction enzyme digestion. *Eco*R I and *Bam* H I cut at several bases away from the insertion site. Therefore, the digestion product should be a bit larger than the exon fragment. More exactly, the digestion product for exon 3 was 612 bp and that for exon 2 was 142 bp (**Figure 3.6**).

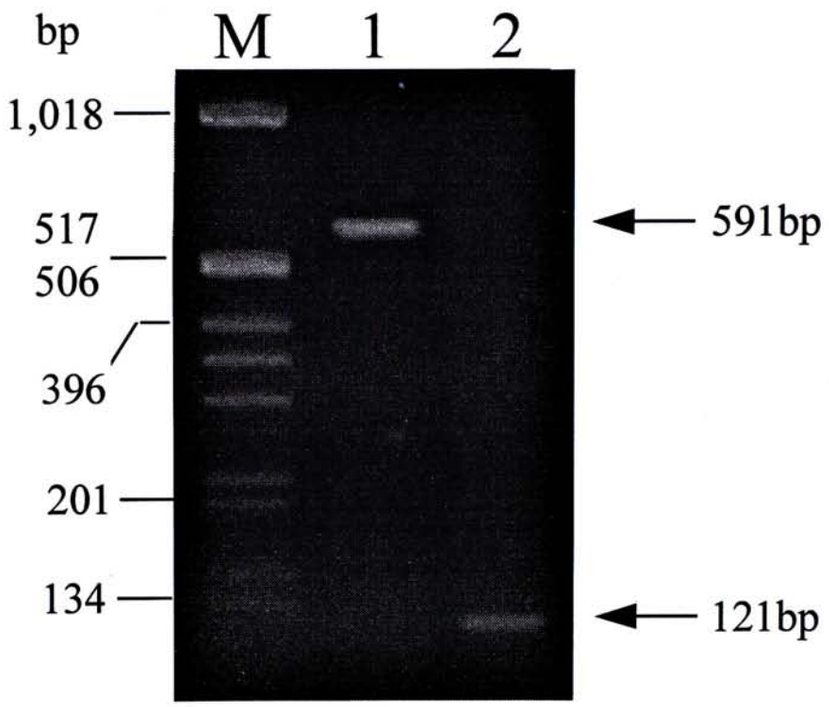


Figure 3.4 PCR products amplified by specific primers for human OSM exons 2 and 3. After 30 cycles of amplification, the products were resolved on Synergel-agarose gel and stained with EtBr. Lane **M**: 1 kb DNA size marker; lane **1**: exon 3 fragment (591 bp); lane **2**: exon 2 fragment (121 bp).

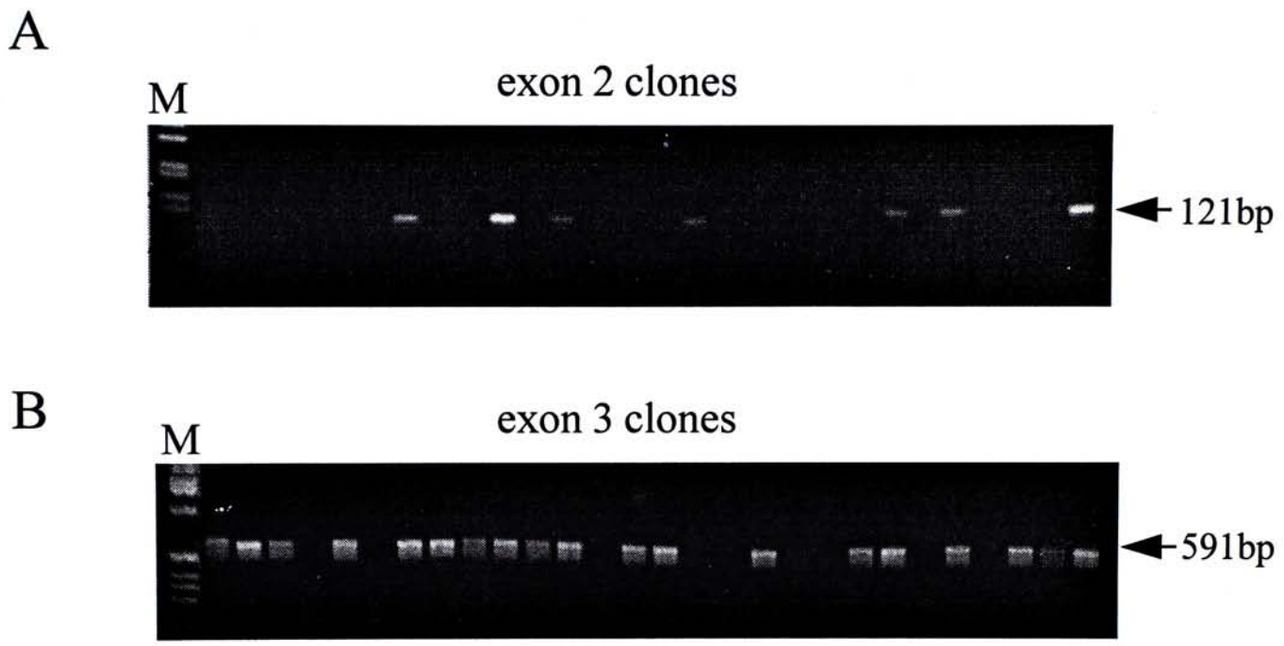


Figure 3.5 Screening of recombinants by PCR. Reaction was performed directly from bacterial colonies which were white in color on LB^{AMP}-X-gal-IPTG plate. Bacterial cells were heat lysed by boiling and subjected to 50-cycle PCR using the original pair of primer either specific to hOSM exon 2 or exon 3. Panel **A**: human OSM exon 2 clones; panel **B**: human OSM exon 3 clones. **M**: 1kb DNA size marker.

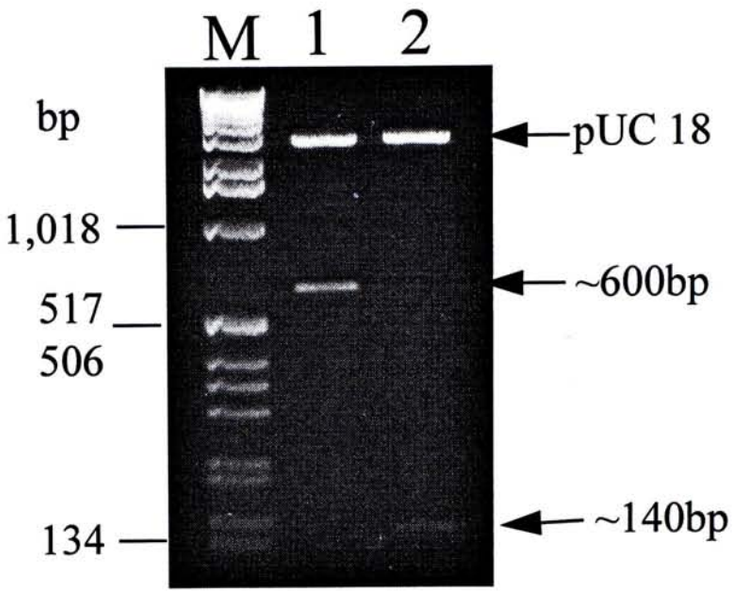


Figure 3.6 Double digestion of recombinant plasmids with *Eco*R I and *Bam* H I. Recombinant plasmids carrying human OSM exon 2 or 3 were digested with the two restriction enzymes. Lane **1**: exon 3 clone. Digestion resulted in a fragment of 612 bp and the vector pUC 18 fragment (2,665 bp). Lane **2**: exon 2 clone. The cut fragment size was 142 bp in addition to the vector band. **M**: 1 kb DNA size marker.

3.4.3 Sequence of subcloned exons 2 and 3

After being screened by PCR and restriction enzyme cutting, the identity of the putative clones was verified by sequencing. The DNA sequences of the two subclones were compared against the Genbank nucleotide and protein databases via the NCBI BLAST Electronic-mail service. The sequences were found to be best fit to human OSM exon 2 and exon 3 fragment, the region flanked by the upper and lower primers. Alignment of putative clone sequences with exon 2 and exon 3 was done to check the similarity (**Figure 3.7**). The alignment test revealed that the PCR cloned human exon 2 and 3 only had 4.35% and 5.74% dissimilarity from the published data respectively. This was most probably due to the errors generated during PCR reaction in which the fidelity of Thermoprime^{plus} DNA polymerase was concerned. Furthermore, error generated during DNA sequencing might have its contribution to the mismatch. In spite of the minor differences, it is reasonable to confirm the identities of the subcloned fragments. In other words, human OSM exons 2 and 3 were successfully cloned by PCR method using the two pairs of specially designed primer. The two verified clones were subsequently used to generate probes of human exon 2 and 3 fragments for hybridization.

Exon 2

```
U   21 TCTGGTGCTTGCACTCTG..TTCCAAGCATGGCGAGCATGGC.GCTATAG 67
       |||||| |||||||||    |||||||||||||||||||||| |||||||
L    8 TCTGGTCCTTGCACTCCTGTTTCCAAGCATGGCGAGCATGGCGGCTATAG 57

U   68 GCAGCTGCTTG.AAGAGTACCGCGTGCT.CTTGGCCAACTCCAGAAGCAG 115
       ||||||||| | |||||||||||||||| |||||||| ||||||||||||
L   58 GCAGCTGCTCGAAAGAGTACCGCGTGCTCCTTGGCCAGCTCCAGAAGCAG 107

U  116 ACAGATCTCATGCAGGACAC 135
       ||||||||||||||||||||
L  108 ACAGATCTCATGCAGGACAC 127
```

% Similarity: 95.65

Exon 3

```
U   15 CCAAGGCCTGGATGTTCCTAAACTGAGAGAGCACTGCAGGGAGCGCCC.. 62
       ||||||||||||||||||||||||||||||||||||||||||||||||||
L   15 CCAAGGCCTGGATGTTCCTAAACTGAGAGAGCACTGCAGGGAGCGCCCCG 64

U   63 GGGCCTT.CCCAGTGAGGAGA.CCTGAGGGG..CTGGCAGGCGGGG.TTC 107
       ||||||| |||||||||||||| |||||||||    |||||||||||| |||
L   65 GGGCCTTCCCCAGTGAGGAGACCCTGAGGGGGCTGGGCAGGCGGGGCTTC 114

U  108 CTGAAGACCCTCAATGCCACACTGGGCTGCGTCCTGAACAGACTTGCCCG 157
       ||| ||||||||||||||||||||||||||||||| |||||| || |||
L  115 CTGCAGACCCTCAATGCCACACTGGGCTGCGTCCTGCACAGAC.TGGCCG 163

U  158 ACTTAGAGCAGCGCCTCCCCAAGGCCCAGGATTTGGAGGAGGCTGGGCTG 207
       |||||||||||||||||||||||||||||||||||  | ||||||||
L  164 ACTTAGAGCAGCGCCTCCCCAAGGCCCAGGATTTGGAGAGGTCTGGGCTG 213

U  208 .ACATCCGAGCCTTGGAG 224
        |||||   | |||||||
L  214 AACATCGAGGACTTGGAG 231
```

% Similarity: 94.26

Figure 3.7 Alignment of nucleotide sequences of subcloned PCR product and human OSM exon fragment. Upper (**U**) is the sequence of subcloned PCR product while lower (**L**) is the documented human OSM exon fragment.

3.4.4 Southern hybridization

a. Genomic DNA preparation

Human and mouse genomic DNA were purchased from company while those of BALB/c and C57BL were extracted from their spleens in our laboratory using Xu's protocol (1990). The concentration and yield of DNA were determined by spectrophotometric measurement and were listed in **Table 3.2**:

Table 3.2 Extraction of genomic DNAs of BALB/c and C57BL mouse strains.

	BALB/c	**C57BL**
OD_{260}	0.042	0.024
OD_{280}	0.023	0.012
OD_{260} / OD_{280}	1.857	1.974
Concentration (μg/μl)	0.42	0.24
Yield (μg DNA / spleen)	840	480

Quality of the extracted DNA was checked by agarose gel electrophoresis (**Figure 3.8**). Most of the molecules were greater than 23 kb and RNA contamination was not detected in both cases.

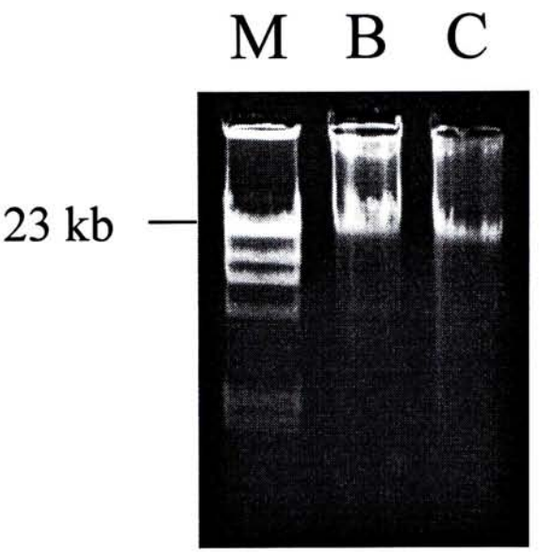


Figure 3.8 Genomic DNA of BALB/c (lane **B**) and C57BL (lane **C**) resolved on 1% agarose gel. Lane **M** is the *Hin*d III cut lambda DNA whose largest DNA band is 23 kb in size.

b. Digestion of genomic DNAs

Large DNA molecules were digested by restriction enzymes before blotted on membrane for hybridization. Human and mouse DNA were cut by three restriction enzymes (*Eco*R I, *Hin*d III and *Pst* I) and the resultant products were resolved in agarose gel as shown in **Figure 3.9**. *Eco*R I, *Hin*d III and *Pst* I chopped DNA into pieces with sizes ranged from several hundred base pairs to 30 kb which appeared as smears on gel during electrophoresis. Satellite DNA bands were observed in *Hin*d III digested human DNA and *Eco*R I digested mouse DNA. It is worth to note that *Hin*d III could not properly digest the mouse DNA molecules because of certain technical problems. Most of the DNA molecules were still greater than 30 kb in size after digestion and clumped together.

In addition, four identical sets of blotting of *Eco*R I digested BALB/c and C57BL genomic DNA were also prepared for Southern hybridization against human OSM exon 2 fragment. Similarly, the products were resolved on agarose gel before blotting and the gel electrophoresis photo was presented in **Figure 3.10**. Satellite DNA bands were also observed in the digest.

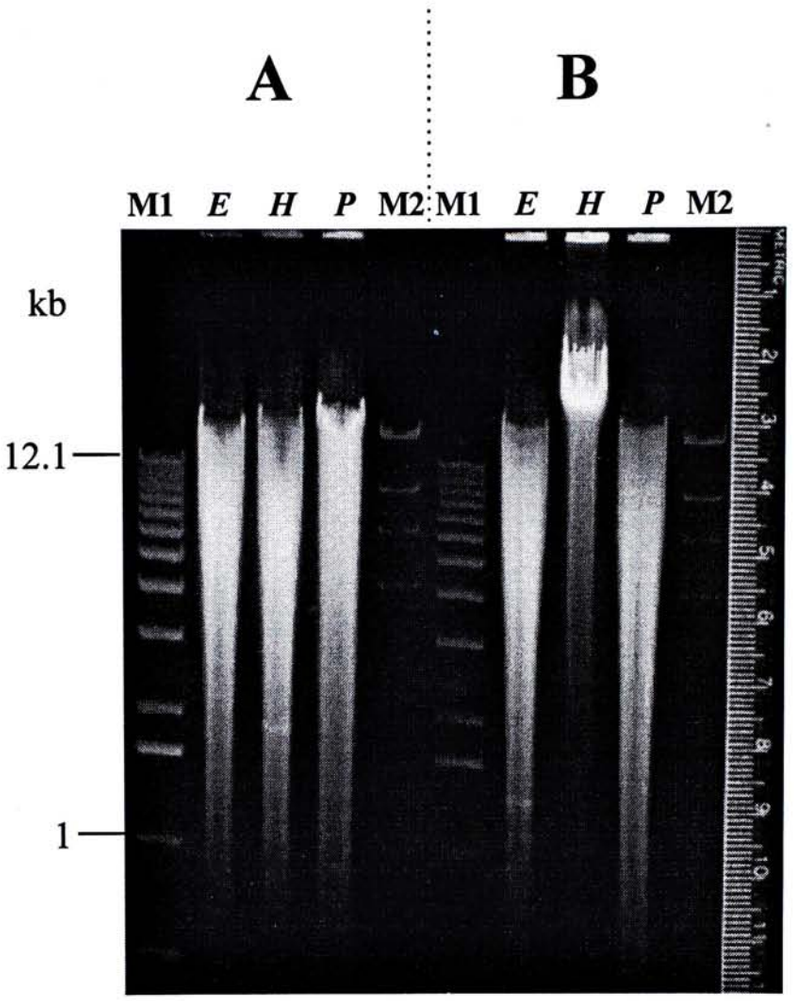


Figure 3.9 Electrophoresis of the restriction enzyme digested human and mouse genomic DNA. 10μg of human and mouse genomic DNA were separately digested with *Eco*R I (lane ***E***), *Hin*d III (lane ***H***), *Pst* I (lane ***P***) and subsequently fractionated on 1% agarose gel.

Panel **A**: human genomic DNA; panel **B**: mouse genomic DNA.

M1 is 1 kb DNA ladder; **M2** is the *Hin*d III cut lambda DNA.

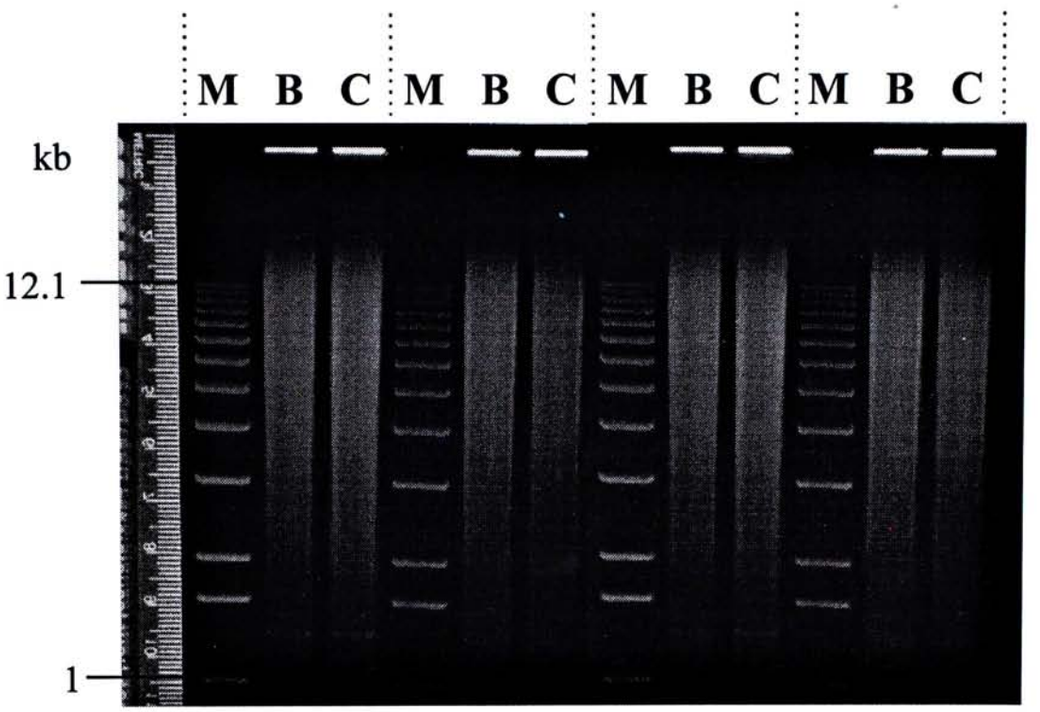


Figure 3.10 Electrophoresis of *Eco*R I digested mouse genomic DNA. Before transferred to nylon membrane, 4 sets of BALB/c (**B**) and C57BL (**C**) mouse genomic DNA were separately digested with *Eco*R I and fractionated on 1% agarose gel. Each set of mouse DNA was accompanied with a size marker, 1 kb DNA ladder (lane **M**), on the left-hand-side.

c. Hybridization signal

After digestion and fractionation on agarose gel, genomic DNAs were transferred to nylon membrane for hybridization. In the first trial, exon 2 fragment was used as probe against mouse genomic DNA. Four identical mouse DNA blots were prepared simultaneously in one blotting step. The four membranes were then subjected to same hybridization conditions but different stringency of washing, from 2X SSC up to 0.1X SSC at 60°C. Hybridization signals were observed in only 2X and 1X blots but not in 0.5X or 0.1X blots as shown in **Figure 3.11**. Three DNA bands, which had migrated 3.9 cm, 4.9 and 5.3 cm were found in both mouse species, but an additional band which migrated 7.5 cm was detected in C57/Black. These DNA molecules binded with the probe, thus having homologous sequences. Their estimated sizes were 6.7 kb, 5.0 kb, 4.5 kb and 2.4 kb respectively. Only the 6.7 kb fragment band was detected in 1X blot. The band became faint as stringency of washing increased.

Exon 3 fragment was then probed against both human and mouse genomic DNA but with different hybridization and washing stringency (referred to *Section 3.3.6 d*). Hybridizing signals were observed in the human blot (**Figure 3.12**). *Eco*R I, *Hin*d III and *Pst* I all gave a single positive band. Their molecule sizes were 5.1 kb, 6.7 kb and 4.3 kb respectively, as derived from their migration distances.

As seen from **Figure 3.13**, positive signal in smears resulted when using human OSM exon 3 fragment as probe against mouse DNA. The situation did not improve even the washing stringency rised to 1X SSC.

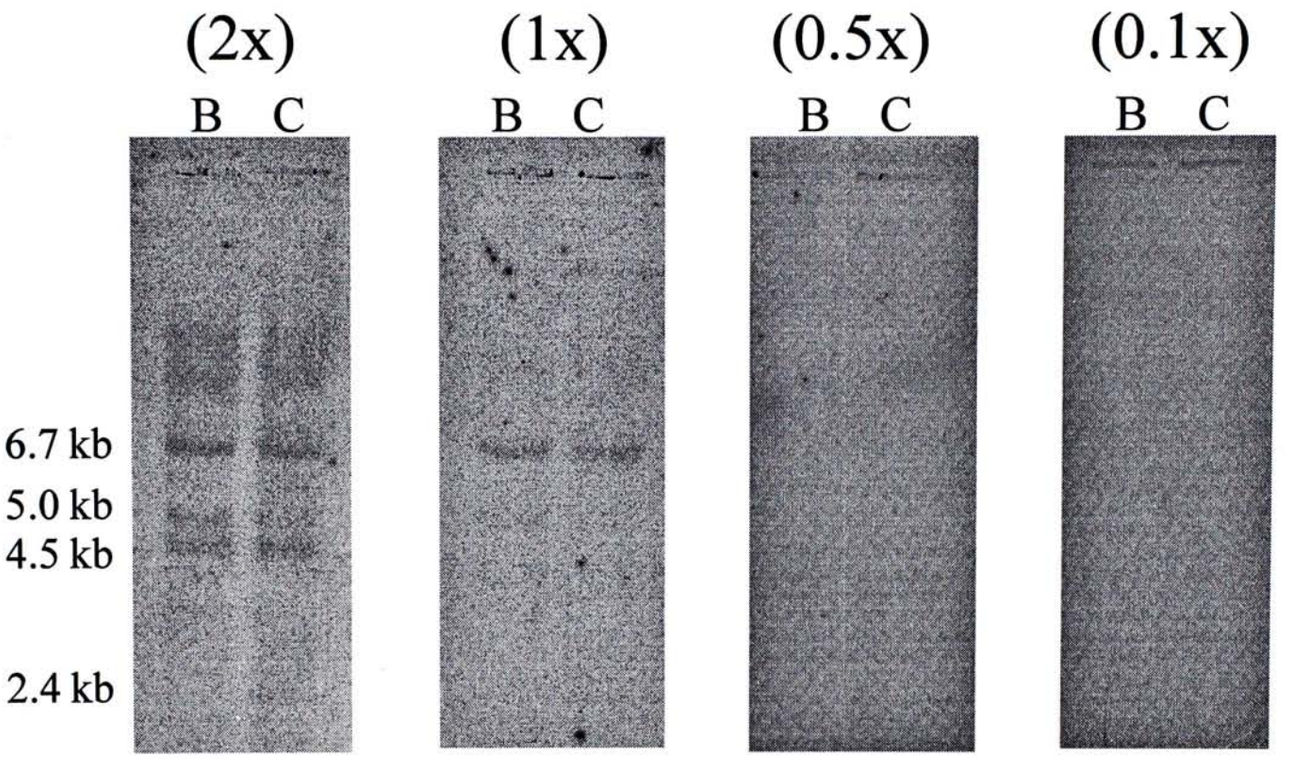


Figure 3.11 Southern hybridization of **mouse** genomic DNAs against human OSM **exon 2** fragment. Four identical blots were prepared for different stringent washing conditions. The number in parenthesis indicates the lowest concentration of SSC used for washing that individual blot. The sizes of the signal bands were determined by measuring their migration distances and referring to a semi-log scale standard curve. Lane **B**: BALB/c DNA; **C**: C57BL DNA

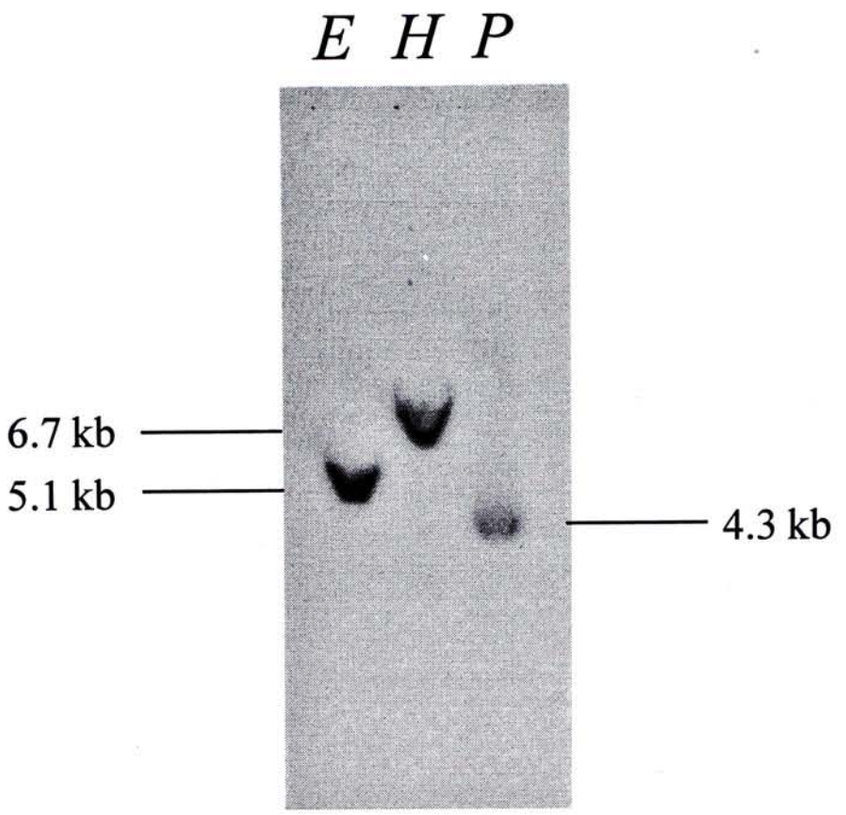


Figure 3.12 Southern hybridization of **human** genomic DNA against human OSM **exon 3** fragment. Restriction enzyme digested human DNAs (as shown in Figure 3.9A) were blotted on membrane and hybridized with radioactively labeled hOSM exon 3 fragment. The hybridization was carried out at 68°C overnight and the washing stringency was up to 0.1X SSC; 0.1% SDS at 68°C. ***E***: *Eco*R I digest gave a positive signal which corresponds to a fragment 5.1 kb in length; while ***H***: *Hin*d III digest and ***P***: *Pst* I digest contained 6.7 kb and 4.3 kb fragments respectively, which are homologous to human OSM exon 3 sequence.

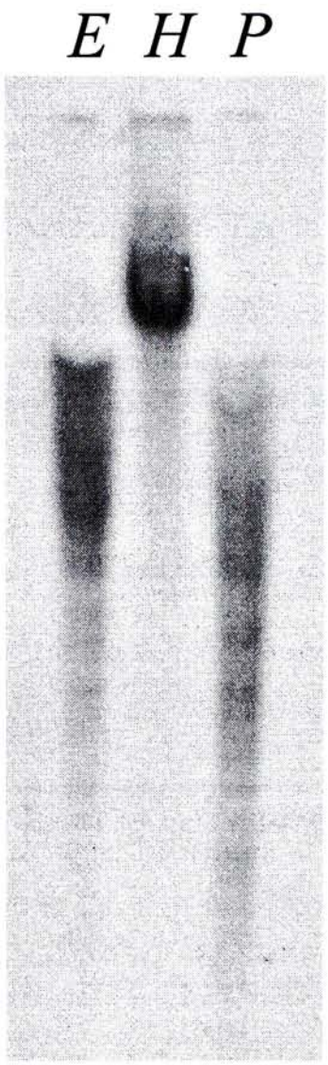


Figure 3.13 Southern hybridization of **mouse** genomic DNA against human OSM **exon 3** fragment. Restriction enzyme digested mouse DNAs (as shown in Figure 3.9B) were blotted on membrane and hybridized with radioactively labeled hOSM exon 3 fragment. The hybridization was carried out at 60°C overnight and the washing stringency was up to 1X SSC; 0.1% SDS at 60°C.

E: *Eco*R I digest; ***H***: *Hind* III digest and ***P***: *Pst* I digest.

3.5 Discussion

3.5.1 Cross-species hybridization

The aim of the second part of the project is to perform preliminary study of the not yet cloned mouse OSM gene so as to check the feasibility of using human OSM as probe for fishing out the mouse counterpart. There were examples of using this type of cross-species hybridization in cloning genes. For instance, human IL-5 gene was isolated using mouse IL-5 cDNA as probe (Campbell *et al.*, 1987). The homology between the coding region of the mouse and human IL-5 gene is found to be 74.9%. In another case, Dr Fung's group attempted to use murine IL-3 coding sequence as probe to isolate the corresponding human IL-3 gene (Dr M.C. Fung, personal communication). However, they failed to obtain the human clone because of the low sequence homology (45%) between the two coding regions (Dorssers *et al.*, 1987). Yang *et al.* (1986), on the other side, could successfully isolate the human IL-3 gene by using gibbon IL-3 cDNA as probe. The success was due in large part to the significant homology between the gibbon and human IL-3 coding region (99.5%).

3.5.2 Hybridization of human OSM exon fragments against mouse genome

a. hOSM exon 2 as probe

Four identical sets of *Eco*R I digested BALB/c and C57BL genomic DNA were blotted on membranes and subjected to hybridization with cloned hOSM exon 2

fragment as probe under the same condition. After hybridization, the four individual membranes were washed separately with different washing stringencies, ranging from 2X SSC up to 0.1X SSC at 60°C. A weak band corresponding to 6.7 kb could be clearly seen in the blots of 2X and 1X SSC washing conditions. Two additional bands of 5.0 kb and 4.5 kb were observed in the 2X SSC washed blot. One more faint band corresponding to 2.4 kb DNA could be seen in C57/Black mouse strain. No hybridization signal was detected under high stringency washing (0.5X and 0.1X SSC).

In general, the washing conditions should be as stringent as possible. A combination of temperature and salt concentration should be determined empirically. In this exon 2 trial, immobilized mouse DNA was hybridized to human OSM exon 2 at 60°C and then washed with buffers of different salt concentrations at 60°C. As the temperature was fixed, the stringency solely depended on the ionic strength which in turn affected the melting temperature (T_m) of the hybrid formed between the probe and its target. T_m may be estimated from the following equation:

$$T_m = 81.5°C + 16.6(\log_{10}[Na^+]) + 0.41(\%G+C) - (600/l)$$ (Maniatis *et al.*, 1982)

where l is the length of the hybrid in base pair and $[Na^+]$ is the concentration of sodium ions in the final stringent wash solution. It should be noted that T_m of a double-stranded DNA decreases by 1 - 1.5°C with every 1% drop in homology (Bonner *et al.*, 1973).

As there are no positive signals found in 0.5 or 0.1X SSC washing, the homology between human and mouse OSM gene is expected to be not high.

b. hOSM exon 3 as probe

Human OSM exon 3 was also used as probe to perform a Southern hybridization against mouse genomic DNA. This time, human genomic DNA was included and done in parallel with the mouse blot but with different hybridization and washing conditions. As human gene exon to human genome, higher stringency of hybridization and washing was carried out for the human blot (hybridized and washed at 68°C and up to 0.1X SSC washing). Single band was detected for *Eco*R I, *Hin*d III and *Pst* I digested human DNA at the same time.

For the mouse blot, washing was carried out with 1X SSC at 60°C. In contrast to the result of using hOSM exon 2 as probe, smears of signal was observed. Such non-specific binding is most likely due to the high G-C content of the hOSM exon 3. The probe used here was the coding region of hOSM exon 3 which contains 65.25% of G and C bases.

c. Feasibility of using hOSM as probe for fishing out the mOSM gene

As viewed from the result of using hOSM exon 2 as probe, a corresponding mouse OSM gene was detected. It is therefore possible to isolate the mouse OSM gene using the human OSM cDNA as probe. However, the percentage of homology between two species OSM gene is quite low, thus rendering the process difficult.

Furthermore, the probe should be selected at a region of lower G-C content so as to avoid non-specific binding during hybridization. Therefore, hOSM exon 3 coding region that has high G-C content could not be used as probe for this purpose.

d. The cloning of mouse OSM by Yoshimura's group

Nevertheless, the murine homologue of OSM has been successfully cloned by Yoshimura's group lately (1996). Their approach of cloning mOSM did not employ cross-species hybridization but a cDNA library subtraction procedure. Since OSM has been identified as a cytokine-inducible immediate early gene through the JAK-STAT5 signaling pathway, mOSM clone was screened out from a subtracted cDNA library in which JAK-STAT5 inducible genes were enriched. The full-length mOSM cDNA was sequenced and the intron-exon structure was also determined. The human and mouse clones are similar in their gene structure, that is three exons with two intervening introns which are found at identical sites (Yoshimura *et al.*, 1996).

By comparing the sequence of human (Malik *et al.*, 1989) and mouse (Yoshimura *et al.*,1996) OSM exon 2, the homology is found to be very low (only 28.16%) within this region. This finding confirms the prediction inferred from the result of the present study.

Chapter 4

Conclusion

4.1 Summary of cytokine and cytokine receptor genes expression during embryonic development

In this part of the project, the expression pattern of 30 cytokines and cytokine receptors over the course of embryonal development in mouse was examined. The results clearly showed that the expression of these molecules were developmentally regulated. Two important points can be drawn from the above observations.

The first point is that the subset of cytokines controlling hematopoiesis in adult is different from those functioning in embryo. Most hematopoietic cytokines were absent from those embryonic tissues studied in this project, like IL-1α, IL-2, IL-3, IL4, IL-5, IL-6, G-CSF and GM-CSF. Among which, IL-3, G-CSF and GM-CSF were the most characterized factors in adult hematopoiesis, however, they did not express in mouse embryos at all. Therefore, it could be concluded that these 3 factors were not critical to fetal hematopoiesis or development. This point was supported by the observations in the studies of Rappolee *et al.* (1988), Schmitt *et al.* (1991) and Kohchi *et al.* (1994). Only the transcripts of IL-1β, SCF, M-CSF and LIF upon the group were detected in embryos. This observation indicates that the pool of cytokines being effective in adult is much bigger than that in fetus. The absence of a number of

cytokines in developing embryos can be explained in two ways which were schematically represented in **Figure 4.1**.

The first explanation is the pleiotropy and redundancy in biological actions of cytokines. Each cytokine exhibits a wide variety of activities and each activity is exhibited by a wide variety of cytokines. The physiological functions of a particular cytokine can be substituted by its surrogates (**Figure 4.1a**). Indeed, the emerging picture in both haematopoietic and embryonic development is one in which the behavior of a stem is not exclusively regulated by a unique factor, but rather is governed by the interplay of several cytokines.

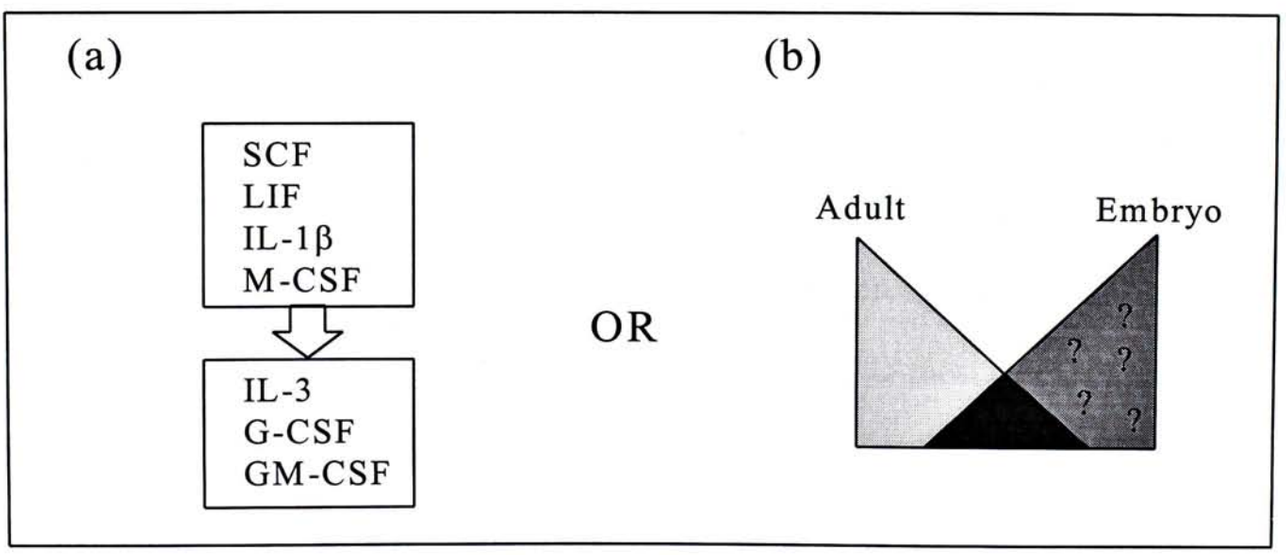


Figure 4.1 Two hypotheses explain why major hematopoietic cytokines were not found in mouse embryos.

An alternative explanation for the smaller pool of cytokines observed in mouse embryo is that there are actually many other factors that have not been discovered yet. These unknown factors may specially control hematopoiesis in embryo, substituting for those cytokines claimed to be important in adult hematopoiesis but absent in embryos. In other words, the subset of cytokines controlling hematopoiesis in adult and in embryos are different with certain overlapping (**Figure 4.1b**). However, further investigation and much more efforts are required to support this hypothesis.

The second point as concluded from the results of the present study is that cytokine receptors can be present even in the absence of their corresponding cytokines. For instance, the transcripts of IL-2Rα, IL-3R(AIC2A) and IL-3R(AIC2B) were detected in all the embryo tissues even though IL-2 and IL-3 were not found in those samples. The biological significance of such phenomenon remains unclear but there are two speculations. One is that expression of cytokine receptors on target cells always precede their corresponding factor. This serves to prepare target cells at a ready state to be responsive to cytokines once they are produced. Schmitt and co-workers (1991), using murine ES cell system, detected the expression of various cytokines and cytokine receptors in differentiated ES cells over 28 days. In most cases, the receptor genes were found to be transcribed before the corresponding cytokines. Another speculation is that the expression of receptors is actually for the binding to certain undiscovered ligands. However, such putative ligands still await our exploration in the future.

4.2 Further studies of the cytokine actions on embryogenesis

The number of cytokines studied in this project is not sufficient to cover the enormous cytokine network. With primers specific to more other cytokines, the temporal pattern of their expression during embryonic development can be checked with this method. Since cytokines exert their actions through the interaction with specific receptors on target cells, the presence of cytokine will not necessarily guarantee an effect. Therefore, the expression pattern of their respective receptors have to be determined as well.

It is noteworthy that the technique RT-PCR only provides a readout of mRNAs being produced by cells at a given point in time. However, transcription of gene does not always correspond qualitatively or quantitatively to translation. Due to this reason, further experimentation is required to determine whether transcripts are indeed translated and bring about their biological effects. Two approaches have generally been used to detect the production of cytokine proteins; they are either an immunological assay using growth-factor-specific antibodies or a bioassay selected for sensitivity to a particular cytokine present in the medium conditioned by producing cells or in cell extracts.

A direct way to reveal the exact function of a cytokine in development is to interrupt its gene expression. This can be achieved by targeted mutagenesis using homologous recombination to disrupt the cytokine gene in ES cells. Such 'knockout

mice' model provides a means of understanding the pathophysiological consequences of the loss of a cytokine, from which the *in vivo* functions of that cytokine can be inferred.

It will be of interest to find out whether cytokines in other mammals, like human, share the same properties. If so, cytokines will not only provide a means to control pregnancy, but it may also help to improve the establishment of successful pregnancies from embryos produced by *in vitro* fertilization.

4.3 Molecular analysis of mouse OSM gene

As viewed from the result of using hOSM exon 2 as probe, a corresponding homologue was detected in the mouse genome. However, the signal was weak and could hardly be seen in high stringency washing. The degree of homology was therefore predicted to be very low.

Using hOSM exon 3 coding region as probe against mouse genomic DNA had resulted into much non-specific bindings, probably due to high G-C content within the region. As concluded from these observations, it will be technically impossible to clone the mouse OSM gene using cross-species hybridization.

The cloning of murine OSM gene will certainly foster the understanding of this cytokine using the mouse model. With mOSM sequence being revealed, specific primers can then be designed and its expression profile during embryogenesis can be determined in the same manner as described in this study. It is expected in the near future that the creation of mice lacking the OSM gene will provide a clear insight into the physiological function of this cytokine.

References

Abbas, A.K., Lichtman, A.H. and Pober J.S. (1991). Cytokines. In: *Cellular and Molecular Immunology*. Wonsiewicz, M.J. eds. pp. 225-243. W.B. Saunders Company, USA.

Adamson, E.D. and Meek, J. (1984). The ontogeny of epidermal growth factor receptors druing mouse development. *Developmental Biology* **103**, 62-70.

Aggarwal, B.B. and Pocsik, E. (1992). Cytokines: From clone to clinic. *Achives of Biochemistry and Biophysics* **292**, 335-359.

Anderson, D.M., Lyman, S.D., Baird, A., Wignall, J.M., Eisenman, J., Rauch, C., March, C.J., Boswell, H.S., Gimpel, S.D., Cosman, D. and Williams, D.E. 1990. Molecular cloning of mast cell growth factor, a hematopoietin that is active in both membrane bound and soluble forms. *Cell* **63**, 235-243.

Bhatt, H., Brunet, L.J. and Stewart, C.L. (1991). Uterine expression of leukemia inhibitory factor coincides with the onset of blastocyst implantation. *Proceedings of the Natural Academy of Sciences USA* **88**, 11408-11412.

Bonner, T.I., Brenner, B.R., Neufeld, B.R. and Britten, R. J. (1973). Reduction in the rate of DNA reassociation by sequence divergence. *Journal of Molecular Biology* **81**, 123.

Bradley, A., Ramirez, S.R., Zheng, H., Hasty, P. and Davis, A. (1992). Genetic manipulation of the mouse via gene targeting in embryonic stem cells. *Ciba Foundation Symposium* **165**, 256-269.

Bruce, A.G., Linsley, P.S. and Rose, T.M. (1992). Oncostatin M. *Progress in Growth Factor Research* **4**, 157-170.

Campbell, H.D., Tucker, W.Q.J., Hort, Y., Martinson, M.E., Mayo, G., Clutterbuck, E.J., Sanderson, C.J. and Young, I.G. (1987). Molecular cloning, nucleotide sequence, and expression of the gene encoding human eosinophil differentiation factor (interleukin 5). *Proceedings of the Natural Academy of Sciences USA* **84**, 6629-6633.

Cao, X., Kozak, C.A., Liu, Y.J., Noguchi, M., O'Connell, E. and Leonard, W.J. (1993). Characterization of cDNAs encoding the murine interleukin 2 receptor (IL-2R) γ chain: Chromosomal mapping and tissue specificity of IL-2R γ chain expression. *Proceedings of the Natural Academy of Sciences USA* **90**, 8464-8468.

Chabot, B., Stephenson, D.A., Chapman, V.M., Besmer, P. and Bernstein, A. (1988). The proto-oncogene *c-kit* encoding a transmembrane tyrosine kinase receptor maps to the mouse *W* locus. *Nature* **335**, 88-89.

Chelly, J. and Kahn, A. (1994). RT-PCR and mRNA quantitation. In: *The Polymerase Chain Reaction*. Mullis, K.B., Ferré, F. and Gibbs, R.A. eds. pp.97-109. Birkhäuser Boston.

Clemens, M.J. (1991). Introduction to cytokines. In: *Cytokines*. Read, A.P. and Brown, T. eds. pp. 1-19. BIOS Scientific Publishers Limited. Oxford.

Conover, J.C., Ip, N.Y., Poueymirou, W.T., Bates, B., Goldfarb, M.P., DeChiara, T.M. and Yancopoulos, G.D. (1993). Ciliary neurotrophic factor maintains the pluripotentiality of embryonic stem cells. *Development* **119**, 559-565.

Conquet, F. and Brulet, P. (1990). Developmental expression of myeloid leukemia inhibitory factor gene in preimplantation blastocysts and in extraembryonic tissue of mouse embryos. *Molecular and Cellular Biology* **10**, 3801-3805.

Dallman, M.J. and Porter, C.G. (1991). Semi-quantitative PCR for the analysis of gene expression. In: *PCR A practical approach*. McPherson, M.J., Quirte, P. and Taylor, G.R. eds. pp.215-224. Oxford University Press, New York.

Das, M., Rengaraju, M. and Samanta, A. (1992). Epidermal growth factor. In: *Human cytokines-Handbook for basic and clinical research*. Aggarwal, B.B. and Gutterman, J.U. eds. pp. 365-382. Blackwell Scientific Publications. USA.

Deuel, T.F. and Kawahara, R.S. (1992). Platelet-derived growth factor. In: *Human cytokines-Handbook for basic and clinical research*. Aggarwal, B.B. and Gutterman, J.U. eds. pp. 300-328. Blackwell Scientific Publications. USA.

Dinarello, C.A. (1994). The interleukin-1 family: 10 years of discovery. *The FASEB Journal* **8**, 1314-1325.

Doherty, A.S., Temeles, G.L. and Schultz, R.M. (1994). Temporal Pattern of IGF-I expression during mouse preimplantation embryogenesis. *Molecular Reproduction and Dvelopment* **37**, 21-26.

Dorssers, L., Burger, H., Bot, F., Delwel, R., Geurts van Kessel, H.M., Lowenbery, B. and Wagemaker, G. (1987). Characterization of a human multilineage-colony-stimulating factor cDNA clone identified by a conserved noncoding sequence in mouse interleukin-3. *Gene* **55**, 115-124.

Evans, M.J. and Kaufman, M.H. (1981). Establishment in culture of pluripotential cells from mouse embryos. *Nature* **292**, 156-158.

Fann, M.J. and Patterson, P.H. (1994). Neuropoietic cytokines and activin A differentially regulate the phenotype of cultured sympathetic neurons. *Proceedings of the National Academy of Sciences USA*. **91**, 43-47.

Fiorentino, D.F., Bond, M.W. and Mosmann, T.R. (1989). Two types of mouse helper T cell. IV. Th2 clones secrete a factor that inhibits cytokine production by Th1 clones. *Journal of Experimental Medicine* **170**, 2081-2095.

Flanagan, J.G., Chan, D.C. and Leder, P. (1991). Transmembrane form of the *kit* ligand growth factor is determined by alternative splicing and is missing in the Sl^d mutant. *Cell* **64**, 1025-1035.

Gearing, D.P., Comeau, M.R., Friend, D.J., Gimpel, S.D., Thut, C.J., McGourty, J., Brasher, K.K., King, J.A., Gillis, S., Mosley, B., Ziegler, S.F. and Cosman, D. (1992). The IL-6 signal transducer, gp130: an Oncostatin M receptor and afffinity convertor for the LIF receptor. *Science* **255**, 1434-1437.

Gearing, D.P., Gough, N.M., King, J.A., Hilton, D.J., Nicola, N.A., Simpson, R.J., Nice, E.C., Kelso, A. and Metcalf, D. (1987). Molecular cloning and expression of cDNA encoding a murine myeloid leukaemia inhibitory factor (LIF). *EMBO Journal* **6**, 3995.

Goodwin, R.G., Friend, D., Ziegler, S.F., Jerzy, R., Falk, B.A., Gimpel, S., Cosman, D., Dower, S.K., March, C.J., Namen, A.E., and Park, L.S. (1990). Cloning of the human and murine interleukin-7 receptors: Demonstration of a soluble form and homology to a new receptor superfamily. *Cell* **60**, 941-951.

Gospodarowicz, D. (1992). Fibroblast growth factor. In*: Human cytokines-Handbook for basic and clinical research.* Aggarwal, B.B. and Gutterman, J.U. eds. pp. 329-352. Blackwell Scientific Publications. USA.

Gough, N.M., Gearing, D.P., King, J.A., Willson, T.A., Hilton, D.J., Nicola, N.A. and Metcalf, D. (1988). Molecular cloning and expression of the human homologue of the murine gene encoding myeloid leukaemia-inhibitory factor. *Proceedings of National Academy Sciences USA* **85**, 2623-2627.

Hamblin, A.A. (1993). Cytokines one by one. In *Cytokines and cytokine receptors*. Rickwood, D. and Male, D. eds. pp. 21-40. Oxford University Press Ind., New York.

Hara,T. and Miyajima, A. (1992). Two distinct functional high affinity receptors for mouse interleukin-3 (IL-3). *The EMBO Journal* **11**, 1875-1884.

Haselbacher, G.K., Schwab, M.E., Pasi, A. And Humbel, R.E. (1985). Insulin-like growth factor II (IGF-II) in human brain: regional distribution of IGF-II and higher molecular mass forms. *Proceedings of the Natural Academy of Sciences USA* **82**, 2153-2157.

Heike, T., Nishikomori, R., Kawai, M., Tsuboi, A., Arai, N. and Mikawa, H. 1994. Developmental changes of GM-CSF gene inducibility in embryonal carcinoma cells. *Molecular Immunology* **31**, 1269-1275.

Heine, U.I., Munoz, E.F., Flanders, K.C., Ellingsworth, L.R., Lam, H.-Y.P., Thompson, N.L., Roberts, A.B. and Sporn, M.B. (1987). Role of transforming growth factor-β in the development of the mouse embryo. *Journal of Cell Biology* **105**, 2861-2876.

Hogan, B., Beddington, R., Costantini, F. and Lacy, E. (1994). Summary of mouse development. In: *Manipulating the mouse embryo. A laboratory manual. Second Edition*. pp.19-105. Cold Spring Harbor Laboratory Press, USA.

Iscove, N.N. and Roitsch, C. 1985. The multilineage hematopoietic growth factors. In: *Cellular and Molecular Biology of Lymphokines*. Sorg, C. and Schimple, A. eds. pp.397-424. Academic Press, New York.

Janeway, C.A. Jr. and Travers, P. (1994). Cytokines can act locally or at a distance. In: *ImmunoBiology - The immune system in health and disease*. Robertson, M., Ward, R. and Lawrence, E. eds. pp.7.17-7.18, A9-A10. Blackwell Scientific Publication, Oxford.

Jessell, T.M. and Melton, D.A. (1992). Diffusible factors in vertebrate embryonic induction. *Cell* **68**, 257-270.

Köhler, T.H. (1995). General aspects and chances of nucleic acid quantitation by PCR. In: *Quantitation of mRNA by Polymerase Chain Reaction*. Köhler, T.H., Laßner, D., Rost, A.K., Thamm, B., Pustowoit, B. and Remke, H. eds. pp.3-14. Springer-Verlag Berlin Heideberg.

Kallestad, J.C., Shoyab, M. and Linsley, P.S. (1991). Disulfide bond assignment and identification of regions required for functional activity of Oncostatin M. *Journal of Biological Chemistry* **266**, 8940-8945.

Kaupmann, K., Sendtner, M., Stockli, K.A. and Jockusch, H. (1991). The gene for ciliary neurotrophic factor (CNTF) maps to murine chromosome 19 and its expression is not affected in the hereditary motoneuron disease 'wobbler' of the mouse. *European Journal of Neurosciences* **3**, 1182-1186.

Kinoshita, T., Imamura, J., Nagai, H. and Shimotohno, K. (1992). Quantification of gene expression over a wide range by the polymerase chain reaction. *Analytical Biochemistry* **206**, 231-235.

Kishimoto, T., Taga, T. and Akira, S. (1994). Cytokine signal transduction. *Cell* **76**, 253-262.

Kohchi, C., Noguchi, K., Tanabe, Y., Mizuno, D. and Soma, G. (1994). Constitutive expression of TNF-α and -β genes in mouse embryo: roles of cytokines as regulator and effector on development. *International Journal of Biochemistry* **26**, 111-119.

Koller, B.H. and Smithies, O. (1992). Altering genes in animals by gene targeting. *Proceedings of the Natural Academy of Sciences USA* **89**, 6070-6074.

Kono, T., Doi, T., Yamada, G., Hatakeyama, M., Minamoto, S., Tsudo, M., Miyasaka, M., Miyata, T. and Taniguchi, T. (1990). Murine interleukin 2 receptor β chain: Dysregulated gene expression in lymphoma line EL-4 caused by a promoter insertion. *Proceedings of the Natural Academy of Sciences USA* **87**, 1806-1810.

Kuby, J. (1994). Cytokines. In: *Immunology*. pp. 297-322. W.H. Freeman and Company. USA.

Kulkarni, A.B., Huh, C.G., Becker, D., Geiser, A., Light, M., Flanders, K.C., Roberts, A.B., Sporn, M.B., Ward, J.M. and Karlsson, S. (1993). Transforming growth factor-β1 null mutation in mice causes excessive inflammatory response and early death. *Proceedings of the Natural Academy of Sciences USA* **90**, 770-774.

Kurzrock, R., Estrov, Z., Wetzler, M., Gutterman, J.U. and Talpaz, M. (1991). LIF: Not just a Leukemia Inhibitory Factor. *Endocrine Reviews* **12**, 208-217.

Lam, A., Fuller, F., Miller, J., Kloss, J., Manthorpe, M., Varon, S. and Cordell, B. (1991). Sequence and structural organization of the human gene encoding ciliary neurotrophic factor. *Gene* **102**, 271-276.

Lee, F.D. (1992). The role of interleukin-6 in development. *Developmental Biology* **151**, 331-338.

Lehnert, S.A. and Akhurst, R.J. (1988). Embryonic expression pattern of TGF-β type 1 RNA suggests both paracrine and autocrine mechanisms of action. *Development* **104**, 263-273.

Malik, N., Kallestad, J.C., Gunderson, N.L., Austin, S.D., Neubauer, M.G., Ochs, V., Marquardt, H., Zaling, J.M., Shoyab, M., Wei, C.M., Linsley, P.S. and Rose, T.M. (1989). Molecular cloning, sequence analysis, and functional expression of a novel growth regulator, oncostatin M. *Molecular and Cellular Biology* **9**, 2847-2853.

Martin, F.H., Suggs, S.V., Langley, K.E., Lu, H.S., Ting, J., Okino, K.H., Morris, C.F., McNiece, I.K., Jacobsen, F.W., Mendiaz, E.A., Birkett, N.C., Smith, K.A., Johnson, M.J., Parker, V.P., Flores, J.C., Patel, A.C., Fisher, E.F., Erjavec, H.O., Herrera, C.J., Wypych, J., Sachdev, R.K., Pope, J.A., Leslie, I., Wen, D., Lin, D.H., Cupples, R.L. and Zsebo, K.M. 1990. Primary structure and functional expression of rat and human stem cell factor DNAs. *Cell* **3**, 203-211.

Massague, J., Kelly, B. And Mottola, C. (1985). Stimulation by insulin-like growth factors is required for cellular transformation by TGF-β. *Journal of Biological Chemistry* **260**, 4551-4554.

Masu, Y., Wolf, E., Holtmann, B., Sendtner, M., Brem, G. and Thoenen, H. (1993). Disruption of the CNTF gene results in motor neuron degeneration. *Nature* **365**, 27-32.

Matsui, Y., Zsebo, K.M. and Hogan B.L.M. (1990). Embryonic expression of a haematopoietic growth factor encoded by the *SI* locus and the ligand for *c-kit*. *Nature* **347**, 667-669.

Metcalf, D., Johnson, G.R. and Burgess, A.W. 1980. Direct stimulation by purified GM-CSF of the proliferation of multipotential and erythroid precursor cells. *Blood* **55**, 138-147.

Migliaccio, D., Migliaccio, R., Valinsky, J., Longley, K., Zsebo, K., Visser, J.W. and Adamson, J.W. (1991). Stem cell factor induces proliferation and differentiation of highly enriched murine haematopoietic cells. *Proceedings of the Natural Academy of Sciences USA* **88**, 7420-7424.

Miles, S.A., Martinez-Maza, O., Rezai, A., Magpantay, L., Kishimoto, T., Nakamura, S., Radka, S.F. and Linsley, P.S. (1992). Oncostatin M as a potent mitogen for AIDS-Kaposi's sarcoma-derived cells. *Science* **255**, 1432-1434.

Moore, M.A.S. and Metcalf, D. 1970. Ontogeny of the haemopoietic system: yolk sac origin of *in vivo* and *in vitro* colony forming cells in the developing mouse embryo. *British Journal of Haematology* **18**, 279-296.

Mosley, B., Beckmann, M.P., March, C.J., Idzerda, R.L., Gimpel, S.D., Vandenbos, T., Friend, D., Alpert, A., Anderson, D., Jackson, J., Wignall, J.M., Smith, C., Gallis, B., Sims, J.E., Urdal, D., Widmer, M.B., Cosman, D. and Park, L.S. (1989). The murine interleukin-4 receptor: molecular cloning and characterization of secreted and membrane bound forms. *Cell* **59**, 335-348.

Mosmann, T.R. and Coffman, R.L. (1989). TH1 and TH2 cells: Different patterns of lymphokine secretion lead to different functional properties. *Annual Review of Immunology* **7**, 145-173.

Mummery, C.L. and van den Eijnden-van Raaij, A.J.M. (1990). Growth factors and their receptors in differentiation and early murine development. *Cell Differentiation and Development* **30**, 1-18.

Murphy, L.D., Herzog, C.E., Rudick, J.B., Fojo, A.T. and Bates, S.E. (1990). Use of polymerase chain reaction in the quantitation of mdr-1 gene expression. *Biochemistry* **29**, 10351-10356.

Murray, R., Lee, F. and Chiu, C. (1990). The genes for leukemia inhibitory factor and interleukin-6 are expressed in mouse blastocysts prior to the onset of hemopoiesis. *Molecular and Cellular Biology* **10**, 4953-4956.

Musashi, M., Yang, Y., Paul, S.R., Clark, S.C., Sudo, T. and Ogawa, M. (1991). Direct and synergistic effects of interleukin-11 on murine hemopoiesis in culture. *Proceedings of the Natural Academy of Sciences USA* **88**, 765-769.

Nakayama, N., Hatake, K., Miyajima, A., Arai, K. and Yokota, T. 1989. Colony-stimulating factors, cytokines and hematopoiesis. *Current Opinion in Immunology* **2**, 68-77.

Nicola, N.A., Metcalf, D., Matsumota, M. and Johnson, G.R. 1983. Purification of a factor inducing differentiation in murine myelomonocytic leukemia cells: Identification as granulocyte colony-stimulating factor (G-CSF). *Journal of Biological Chemistry* **258**, 9017-9023.

Ogawa, M., Matsutaki, Y., Sudo, T., Kira, T., Nakauchi, H. and Nishikawa, S.I. (1991). Expression and function of *c-kit* in hemopoietic progenitor cells. *Journal of Experimental Medicine* **174**, 63-71.

Palacios, R. and Nishikawa, S.I. (1992). Developmentally regulated cell surface expression and function of *c-kit* receptor during lymphocyte ontogeny in the embryo and adult mice. *Development* **115**, 1133-1147.

Patterson, P.H. (1994). Leukemia inhibitory factor, a cytokine at the interface between neurobiology and immunology. *Proceedings of the National Academy of Sciences USA*. **91**, 7833-7835.

Paul, W.E. and Ohara, J. 1987. B-cell stimulatory factor-1/interleukin-4. *Annual Review of Immunology* **5**, 429-459.

Popliker, M., Schatz, A., Avivi, A., Ullrich, A., Schlessinger, J. and Webb, C.G. (1987). Onset of endogenous synthesis of epidermal growth factor in neonatal mice. *Developmental Biology* **119**, 38-44.

Rappolee, D.A., Brenner, C.A., Schultz, R., Mark, D. and Werb, Z. (1988). Developmental expression of PDGF, TGF-α and TGF-β genes in preimplantation mouse embryos. *Science* **241**, 1823-1825.

Regenstreif, L.J. and Rossant, J. 1989. Expression of the *c-fms* proto-oncogene and of the cytokine, CSF-1, during mouse embryogenesis. *Developmental Biology* **133**, 284-294.

Rennick, D., Jackson, J., Yang, G., Wideman, J., Lee, F. and Hudak, S. (1989). Interleukin-6 interacts with interleukin-4 and other hematopoietic growth factors to selectively enhance the growth of megakaryocytes, erythroid, myeloid and multipotential progenitors cells. *Blood* **73**, 1828-1835.

Rettenmier, C.W., Sacca, R., Furman, W.L., Roussel, M.F., Holt, J.T., Nienhuis, A.W., Stanley, E.R. and Sherr, C.J. (1986). Expression of the human *c-fms* proto-oncogene product (Colony-stimulating Factor-1 receptor) on peripheral blood mononuclear cells and choriocarcinoma cell lines. *Journal of Clinical Investigation* **77**, 1740-1746.

Roberts, A.B. and Sporn, M.B. (1990). The transforming growth factor-betas. In *Peptide Growth Factors and their Receptors*. Sporn, M.B. and Roberts, A.B. eds. Pp.421-472. Springer Verlag, Heidelberg.

Roitt, I.M., Brostoff, J. and Male, D.K. (1993). Cell cooperation in the antibody response. In: *Immunology Third edition*. pp. 7.8-7.13. Mosby-Year Book Europe Ltd. Hong Kong.

Rose, T.M. and Bruce, A.G. (1991). Oncostatin M is a member of cytokine family that includes leukemia-inhibitory factor, granulocyte colony-stimulating factor and interleukin-6. *Proceedings of the National Academy of Sciences USA* **88**, 8641-8645.

Rose, T.M., Lagrou, M.J., Fransson, I., Werelius, B., Delattre, O., Thomas, G., de Jong, P.J., Todaro, G.J. and Dumanski, J.P. (1993). The genes for Oncostatin M (OSM) and leukemia inhibitory factor (LIF) are tightly linked on human chromosome 22. *Genomics* **17**, 136-140.

Rose, T.M., Weiford, D.M., Gunderson, N.L. and Bruce, A.G. (1994). Oncostatin M inhibits the differentiation of pluripotent embryonic stem cells. *Cytokines* **6**, 48-54.

Rose, R., Raines, E.W. and Bowen-Pope, D.F. (1986). The biology of Platelet-derived growth factor. *Cell* **46**, 155-169.

Rothstein, J.L., Johnson, D., DeLoia, J.A., Skowronski, J., Solter, D. and Knowles, B. (1992). Gene expression during preimplantation mouse development. *Genes and Development* **6**, 1190-1201.

Scheuermann, R.H. and Bauer, S.R. (1993). Polymerase chain reaction-based mRNA quantification using an internal standard: Analysis of oncogene expression. *Methods in Enzymology* **218**, 446-473.

Schmitt, R.M., Bruyns, E. and Snodgrass, H.R. (1991). Hematopoietic development of embryonic stem cells *in vitro*: cytokine and receptor gene expression. *Genes and Development* **5**, 728-740.

Sendtner, M., Carroll, P., Holtmann, B., Hughes, R.A. and Thoenen, H. (1994). Ciliary neurotrophic factor. *Journal of Neurobiology* **25**, 1436-1453.

Shaw, G. and Kamen, R. (1986). A conserved AU sequence from the 3' untranslated region of GM-CSF mRNA mediates selective mRNA degradation. *Cell* **46**, 659-667.

Sherr, C.J. Rettenmeier, C.W., Sacca, R., Roussel, M.F., Look, A.T. and Stanley, E.R. (1985). The *c-fms* proto-oncogene product is related to the receptor for the mononuclear phagocyte growth factor, CSF-1. *Cell* **41**, 665-676.

Siebert, P.D. (1993). Quantitative RT-PCR. Methods & Applications; book 3. Clontech Laboratories, Inc.

Smith, A.G., Heath, J.K., Donaldson, D.D., Wong, G.G., Moreau, J., Stahl, M. and Rogers, D. (1988). Inhibition of pluripotential embryonic stem cell differentiation by purified polypeptides. *Nature* **336**, 688-690.

Smith, A.G., Nichols, J., Robertson, M. and Rathjen, P.D. (1992). Differentiation inhibiting activity (DIA/LIF) and mouse development. *Developmental Biology* **151**, 339-351.

Smith, E.P., Sadler, T.W. and d'Ercole, A.J. (1987). Somatomedins/insulin-like growth factors, their receptors and binding proteins are present during mouse embryogenesis. *Development* **101**, 73-82.

Stanley, E.R. and Heard, P.M. 1977. Factors regulating macrophage production and growth. Purification and some properties of the colony stimulating factor. *Journal of Biological Chemistry* **252**, 4305-4312.

Stewart, C.L., Kaspar, P., Brunet, L.J., Bhatt, H., Gadi, I., Kontgen, F. and Abbondanzo, S.J. (1992). Blastocyst implantation depends on maternal expression of leukaemia inhibitory factor. *Nature* **359**, 76-79.

Stewart, C.L. (1994). Leukaemia inhibitory factor and the regulation of pre-implantation development of the mammalian embryo. *Molecular Reproduction and Development* **39**, 233-238.

Theiler, K. (1972). *The House Mouse - Development and Normal Stages from Fertilization to 4 weeks of Age.* pp.1-142. Springer-Verlag Berlin Heidelberg.

Twardzik, D.R. (1985). Differential expression of transforming growth factor-α during prenatal development of the mouse. *Cancer Research* **45**, 5413-5416.

Van Vlasselaer, P. (1995). IL-10 and bone formation/hematopoiesis. In: *Interleukin-10.* deVries, J.E. and de Waal Malefyt, R. eds. pp. 59-68. Springer-Verlag, Heidelberg, Germany.

Wang, A.M., Doyle, M.V. and Mark, D.F. (1989). Quantitation of mRNA by the polymerase chain reaction. *Proceedings of the Natural Academy of Sciences USA* **86**, 9717-9721.

Williams, R.L., Hilton, D.J., Pease, S., Wilson, T.A., Stewart, C.L., Gearing, D.P., Wagner, E.F., Metcalf, D., Nicola, N.A. and Gough, N.M. (1988). Myeloid leukaemia inhibitory factor maintains the developmental potential of embryonic stem cells. *Nature* **336**, 684-687.

Wride, M.A. and Sanders, E.J. (1995). Potential roles for tumour necrosis factor α during embryonic development. *Anatomy and Embryology* **191**, 1-10.

Xu, H.W., Jevnikar, A.M. and Rubin-Kelly, V.E. (1990). A simple method for the preparation of chromosomal DNA from cell culture. *Nucleic Acids Research* **18**, 4943.

Yamasu, K., Onoe, H., Soma, G.I., Oshima, H. and Mizuno, D.I. (1989). Secretion of tumour necrosis factor during fetal and neonatal development of the mouse: ontogenic inflammation. *Journal of Biological Response Modifiers* **8**, 644-655.

Yang, Y.C., Ciarletta, A.B., Temple, P.A., Chung, M.P., Kovacic, S., Witek-Giannotti, J.S., Leary, A.C., Kriz, R. and Clark, S.C. (1986). Human IL-3 (Multi-CSF): Identification by expression cloning of a novel hematopoietic growth factor related to murine IL-3. *Cell* **47**, 3-10.

Yoshida, K., Taga, T., Saito, M., Suematsu, S., Kumanogoh, A., Tanaka, T., Fujiwara, H., Hirata, M., Yamagami, T., Nakahata, T., Hirabayashi, T., Yoneda, Y., Tanaka, K., Wang, W.Z., Mori, C., Shiota, K., Yoshida, N. and Kishimoto, T. (1996). Targeted disruption of gp130, a common signal transducer for the interleukin 6 family of cytokines, leads to myocardial and hematological disorders. *Proceedings of the Natural Academy of Sciences USA* **93**, 407-411.

Yoshimura, A., Ichihara, M., Kinjyo, I., Moriyama, M., Copeland, N.G., Dilbert, D.J., Jenkins, N.A., Hara, T. and Miyajima, A. (1996). Mouse oncostatin M: an immediate early gene induced by multiple cytokines through the JAK-STAT5 pathway. *The EMBO Journal* **15**, 1055-1063.

Zarling, J.M., Shoyab, M., Marquardt, H., Hanson, M.B., Lioubin, M.N. and Todaro, G.J. (1986). Oncostatin M: a growth regulator produced by differentiated histiocytic lymphoma cells. *Proceedings of the National Academy of Sciences USA* **83**, 9739-9743.

Zsebo, K.M., Wypych, J., McNeice, I.K., Lu, H.S., Smith K.A., Karkare, S.B., Sachdev, R.K., Yuschenkoff, V.N., Birkett, N.C., Williams, L.R., Satyagal, V.N., Tung, W., bosselman, R.A., Mendiaz, E.A. and Langley, K.E. (1990). Identification, purification and biological characterization of hematopoietic stem cell factor from buffalo rat liver-conditioned medium. *Cell* **63**, 195-201.